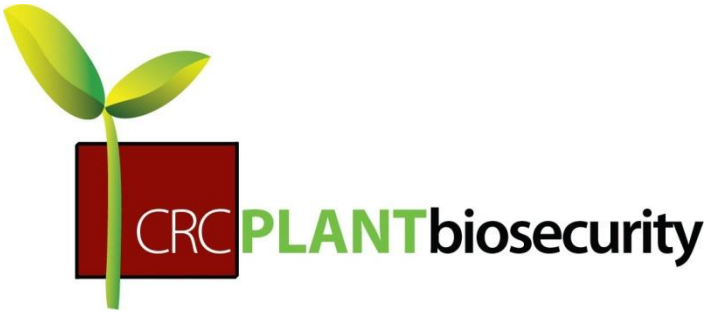

Cooperative Research Centre
for National Plant Biosecurity

Final Report

CRC40050

Enhanced diagnostic platforms for Post Entry Quarantine (PEQ) and market access

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Table of contents

Index of Tables

Index of Figures

1 Executive Summary

The vision of the CRCNPB post-entry quarantine (PEQ) project is to develop advanced molecular diagnostic methods for the detection of plant viruses that can be expediently applied in both a PEQ (import) and a market access (export) context. In phase I of this project (18 months duration), the project team; i) conducted a review of the importation processes of cereals into Australia and New Zealand, ii) designed and validated molecular tests that target groups of plant viruses that pose a threat to the grain's industry and iii) investigated the application of Flinders Technology Associates (FTA) card technologies for rapid and safe collection and storage of plant virus samples and viral ribonucleic acid (RNA).

A review of the importation processes of cereals into Australia and New Zealand identified a need to:

- Review the current 'open quarantine' policy.
- Develop diagnostic tools to support PEQ pathologists, as current screening strategies is dependent on visual inspections, and
- Facilitate the development of closer links between Australian Quarantine and Inspection Service (AQIS) PEQ pathologists and specialist diagnosticians in Australia.

A Reverse-transcription polymerase chain reaction (RT-PCR) assay using a novel set of universal primers for the detection of virus species from the genus *Potyvirus* was validated in the project. The RT-PCR assay was applied to 40 plant samples, some confirmed (via serological and biological tests done previously) to be infected with potyviruses, whilst others were only suspected to be infected with a potyvirus. The assay was successful in detecting potyviruses in all forty amples tested (100% detection rate).

Using a systematic approach to degenerate primer design, diagnostic assays were generated for four virus groups; the furoviruses, hordeiviruses, rymoviruses and tritimoviruses. The diagnostic assays were evaluated against positive controls from each virus group and validation using previously untested samples is now ready to commence.

A quantitative reverse-transcription (RT) polymerase chain reaction (PCR) has been established to assess the quality and rate of decay of viral RNA extracts stored on the FTA cards over time. The quantitative RT-PCR system has been designed to detect Potato virus Y (PVY) and will be used to detect nucleic acid extracted from PVY infected potato leaves. Results will identify a level of confidence for the easy and safe storage of viral nucleic acid on FTA cards.

Research activities are on-going as part of the three year phase II component of this project. It is anticipated that by its completion we will be able to detect one third of all known plant viruses, which includes 14 of the 22 viruses listed under the Emergency Plant Pest Response Deed (EPPRD). The developed tests will be submitted as nationally validated diagnostic protocols (coordinated by the Subcommittee on Plant Health Diagnostic Standards (SPHDS) and utilised in PEQ and during an incursion response. Once adopted by PEQ, new germplasm will be released from PEQ facilities faster and with reduced risk.

2 Aims and objectives

Australia is under constant pressure to allow the importation of fresh produce into Australia. This trend is likely to increase with the implementation of free trade agreements with other countries and the continual growth of the human population. Post entry quarantine (PEQ) underpins the safe importation of plant material such as grains. PEQ diagnostics and border biosecurity in general present enormous challenges to Australia and New Zealand. The identification of an emergency plant pest (EPP) is often based on visual inspections that can be compromised by issues such as latent infection and expression of environmental stresses (e.g., water deficiency). Considerable uncertainty also exists about the pathogens associated with some categories of imported plants (e.g. ornamentals).

There is an urgent need for the development and employment of sensitive, efficient and reliable advanced molecular tests in PEQ to better protect Australia's plant industries. For the grains industry, there is currently no active testing for EPPs of cereals. The reliance on the identification of visual symptoms to detect EPPs is compromised by symptomless infections. The use of pre-described tests for defined lists of pathogens in PEQ is preferred but this strategy does not allow for the detection of unknown strains and/or genera of pathogens.

This project aims to:

i. document the processes for the importation of cereals into Australia and New Zealand through PEQ

ii. validate a generic diagnostic assay for the detection of potyviruses (the largest group of plant viruses known to exist)

iii. develop advanced molecular diagnostic tools for Post Entry Quarantine and for use in incursion response, to support the detection of Emergency Plant Pests in the Australian Grains industries

iv. investigate the feasibility of using FTA cards to assist the transport and storage of plant virus nucleic acids, and

v. document clear directives for phase II funding of this project (2009-2011).

The development of novel strategies for the detection of plant pest groups/genera that have been developed in this project will reduce biosecurity risks from importations of plant material to Australia and New Zealand. The incorporation of new advances in molecular detection, universal primer design and immunological tools into PEQ systems will enhance management of trade and biosecurity risks for the Australian grains industry and the New Zealand and Australian nursery industries.

3 Key findings

3.1 Document the processes for the importation of plant material to Australia and New Zealand through PEQ

Winter cereals comprise the bulk of Australia's grains industry and are the focus of this report. There are several pathways for the introduction of winter cereals into Australia and include importation of seed or clonal grasses through an AQIS approved premises or PEQ station, or via Open Quarantine (seed only). All accessions that enter Australia are fumigated, hot water and fungicide treated. As a result, the Emergency Plant Pests (EPPs) of concern are those pathogens that are seed transmitted (viruses and some bacteria) or resistant to the above treatments. A full documentation of the importation process and critical control points of entry for winter cereals for both Australia and New Zealand is presented in **Appendix A**.

Visual inspections form the basis of the disease screening of cereal seedlings in PEQ approved and open quarantine premises. This can be a difficult task even for experienced pathologists inspecting plants in controlled PEQ facilities, let alone less experienced staff conducting inspections in an open quarantine facility. Access to reliable diagnostic tools to assist quarantine pathologists is urgently required.

Based on our findings from this report we have identified the following opportunities and recommendations to enhance the safe importation of winter cereal germplasm into Australia:

- Develop diagnostics to support PEQ pathologists.
- Facilitate the development of closer links between AQIS PEQ operations and specialist diagnosticians in Australia (mostly employed by state governments) to assist with diagnosis of symptomatic material.
- Conduct detailed analyses of pest lists for winter cereal commodities (as done for pulses), as this data will enable the ICON (AQUIS's import condition database) database to be updated.
- Identify a strategy to constantly update the ICON database for new and emerging pests and diseases that threaten the grains industry.
- Review the open quarantine policy and/or develop a diagnostic capability to support this protocol.
- To increase the biosecurity of the open quarantine protocol, identify pests and pathogens of winter cereals that occur in New Zealand and which have not been recorded in Australia.

An opportunity exists to improve our diagnostic capability to rapidly, accurately and consistently identify pests and diseases intercepted at the barrier.

3.2 Evaluation of potyvirus-specific universal primers

A novel pair of group-specific universal primers for detection of virus species within the *Potyvirus* genus was designed and developed previously (Zheng *et al.*, 2008a; Zheng *et al.*, 2008b). The target sites for this primer set reside in the NIb gene of the potyvirus genomes and were appropriately named NIb2F (sense primer) and NIb3R (antisense primer) (Zheng *et al.*, 2009). These primers were evaluated as part of this project for the detection of potyviruses in both the Victorian Department of Primary Industries (DPIVIC) and the New Zealand (MAF) laboratory. The ability of the NIb2F-3R primers to detect potyviruses was compared to two previously published potyvirus-specific primer sets; WCIEN (Pappu *et al.*, 1993) and Potyvirid (Gibbs *et al.*, 1997).

3.2.1 Vic DPI evaluation of NIb2F-NIb3R primer set for potyvirus detection.

RNA from 44 samples, 40 of which were potyvirus isolates representing 23 recognised and three possible new species was tested (Table 3.1 and Table 3.2). Two virus isolates, one represents *Tritimovirus* and the other representing *Carlavirus* were used as negative controls for the experiment. Two samples previously thought to have potyvirus infection were proved to be negative on all tests by sequencing results.

Reactions with NIb2F and NIb3R produced amplicons of 350bp from all 40 virus isolates tested. Reactions with the previously published WCIEN and Potyvirid primers amplified cDNA from 32 and 21 isolates, representing possibly 21 and 15 species respectively. The identity of 12 unknown potyvirus isolates was confirmed by sequencing and three were found to be potentially distinct and as yet undescribed potyvirus species (Table 3.2).

Gel banding patterns from reactions with NIb2F and NIb3R were simpler to interpret when compared with those from reactions with the other two primer sets; fewer products were visible and the cDNA fragments were less variable in size. RT-PCR with the novel primers is predicted to be able to detect virus isolates from all major groups within the genus *Potyvirus* and the reliability of the PCR test is better suited for use as a routine diagnostic assay. For a more detailed report on the evaluation of the NIb2F-NIb3R primers, see **Appendix B** for the published article (Zheng *et al.*, 2009).

Table 3.1 Virus isolates, associated plant hosts and their RT-PCR results using the NIb2F and NIb3R primers (NIb assay), CN48 and oligo-dT primers (WCIEN assay) and the Potyvirid primers 1 and 2 (Potyvirid assay)

Sample Number	Virus name	Host	Geographic Origin	NIb	WCIEN	Potyvirid
1	Apium virus Y	*Conium maculata*	Angle Crossing, ACT	+	-	+
2	Apium virus Y	*Petroselinum crispum*	Toowoomba, QLD	+	+	+
3	Bean common mosaic virus	*Phaseolus vulgaris*	Biloela, QLD	+	+	-
4	Bean common mosaic virus	*Macroptilium lathyroides*	Anstead, QLD	+	+	-
5	Bean common mosaic virus	Not recorded	Not recorded	+	+	+
6	Bean yellow mosaic virus	*Mandevilla sp.*	Not recorded	+	+	+
7	Carrot virus Y	*Apiaceae*	Not recorded	+	-	-
8	Ceratobium mosaic virus	*Bulbophyllum gradiflorum*	Not recorded	+	+	+
9	Ceratobium mosaic virus	*Grastidium luteocilium*	Not recorded	+	+	+
10	Clover yellow vein virus	*Coriandrum sativum*	Logan Village, QLD	+	+	+
11	Cowpea aphid borne mosaic virus	*Phaseolus vulgaris*	Inglewood, QLD	+	+	-
12	Dasheen mosaic virus	Not recorded	Not recorded	+	+	-
13	Freesia mosaic virus	*Freesia*	Not recorded	+	-	+
14	Johnsongrass mosaic virus	*Zea mays*	Gatton, QLD	+	+	+
15	Papaya ringspot virus	*Cucurbita pepo*	Swan Hill, VIC	+	+	-
16	Papaya ringspot virus	*Cucumis sativus*	Gatton, QLD	+	+	-
17	Passionfruit woodiness virus	*Passiflora edulis*	Not recorded	+	+	+
18 [a]	Peanut mottle virus	*Arachis hypogaea*	Rocky Creek, QLD	-	false positive	-

Sample Number	Virus name	Host	Geographic Origin	NIb	WCIEN	Potyvirid
19	Potato virus Y	*Solanum tuberosum*	Not recorded	+	-	+
20	Sugarcane mosaic virus	*Saccharum officinarum*	Not recorded	+	+	-
21	Sweet potato feathery mottle virus	*Ipomea batatas*	Not recorded	+	+	-
22	Turnip mosaic virus	*Hirschfeldia incana*	Angle Crossing, ACT	+	+	+
23	Turnip mosaic virus	*Brassica pekinensis*	Toowoomba, QLD	+	+	+
24	Watermelon mosaic virus & Zucchini yellow mosaic virus	*Cucumis melo ssp melo*	Gatton, QLD	+	+	-
25	Watermelon mosaic virus	Not recorded	Not recorded	+	+	-
26	Watermelon mosaic virus	*Daucus carota*	Brightview, QLD	+	+	-
27	Zucchini yellow mosaic virus	*Cucurbita pepo*	Giru, QLD	+	+	+
28	Zucchini yellow mosaic virus (strain Passiflora virus Y)	*Passiflora foetida*	Possession Island, QLD	+	+	-
29	Zucchini yellow mosaic virus (strain Passiflora virus Y)	*Vigna unguiculata sesquipedalis*	Darwin, NT	+	+	-
Potyvirus Total				**28/28**	**24/28**	**14/28**
Controls						
30	Wheat streak mosaic virus	*Triticum aestivum*	Horsham, VIC	+	+	-
31	Carnation latent virus	*Dianthus caryophyllus*	Not recorded	-	-	-

ACT= Australian Captial Territory, NT= Northern Territory, VIC= Victoria, QLD= Queensland

[a] Partial sequence obtained from DNA products amplified by WCIEN primers

Table 3.2 Uncharacterised virus isolates, associated plant hosts and their RT-PCR results using the NIb2F and NIb3R primers (NIb assay), CN48 and oligo-dT primers (WCIEN assay) and the Potyvirid primers 1 and 2 (Potyvirid assay) and their virus identity, if known

Isolate Number	Host	Geographic origin	NIb	WCIEN	Potyvirid	Virus Confirmed
32 [a]	*Musa spp.*	Not recorded	+	+	-	Banana bract mosaic virus
33 [a]	*Musa spp.*	Not recorded	+	+	-	Banana bract mosaic virus
34 [a]	*Datura tatula*	Angle Crossing, ACT	+	-	+	Potato virus Y
35 [a]	*Hibbertia scandens*	Bawley Point, NSW	+	+	+	Unknown potyvirus
36 [a,b]	*Allium sativum*	Not recorded	+	+	-	Onion yellow dwarf
37 [a]	*Glycine clandestina*	Nuggan Point, NSW	+	-	-	Unknown potyvirus
38 [b]	*Dendrobium speciosum*	Not recorded	+	+	+	Sarcochilus virus Y
39 [c]	*Vigna unguiculata*	Berrimah, NT	+	-	+	Zucchini yellow mosaic virus
40 [a]	*Passiflora edulis*	Not recorded	+	+	-	Unknown potyvirus
41 [c]	*Commelina spp.*	Rosedale, VIC	+	+	+	Unknown potyvirus
42 [a]	*Wisteria sinensis*	Knoxfield, VIC	+	-	-	Wisteria vein mosaic virus
43 [b]	*Zantedeschia spp.*	Canberra, ACT	-	false positive	-	No virus
44 [a]	*Cucurbita maxima*	Darwin, NT	+	+	+	Zucchini yellow mosaic virus
Potyirus Total			**12/12**	**8/12**	**6/12**	

Partial sequence obtained from DNA products amplified by [a] NIb primers, [b]WCIEN primers and [c]Virid primers

3.2.1.1 Material and methods

Positive virus controls

In the DPIVIC laboratory, the NIb assay was tested against 44 plant samples, of which 29 were potyvirus isolates previously confirmed by either molecular or serological testing, two were tritimovirus and carlavirus positive controls (Table 3.1) and the remaining 13 were possible potyvirus-infected samples (Table 3.2). The NIb2F and NIb3R primer set was tested against these samples, along with two other previously published assays: WCIEN (Pappu *et al.*, 1993) and Potyvirid (Gibbs *et al.*, 1997) (Table 3.3)

RNA extraction

Total RNA from all samples was extracted from approximately 0.1 g of freeze-dried or 0.2g of fresh plant tissues using the RNeasy Plant Mini Kit (Qiagen) and a modified lysis buffer (Mackenzie *et al.*, 1997). RNA extracts were then eluted in a final volume of 50 µL and stored in -20^{o}C until use.

Assay and cycling conditions

All nucleic acid extracts were tested by RT-PCR for the presence of potyvirus RNA using the three sets of universal primers, i.e. the NIb, WCIEN and Potyvirid universal assays. All samples were also tested for the presence of amplifiable host RNA using the NAD primer pair. One-step RT-PCR was performed using Superscript™ III Platinum Taq (Invitrogen) in a 25 µL volume reaction as per the manufacturer's instructions, with 1 µL of total RNA extract. The NIb2F and NIb3R primers were used at a final concentration of 0.5 µM. A reverse transcription step was done at 48^{o}C for 45 minutes and terminated at 94^{o}C for two minutes. PCR amplification immediately followed, with 35 cycles at: 95^{o}C for 45 seconds, 45^{o}C for 45 seconds and 72^{o}C for 45 seconds; a final extension of five minutes was done at 72^{o}C and the reactions were then held at room temperature. Primer concentrations and PCR cycling conditions for the WCIEN, Potyvirid and NAD assays were as previously described (Pappu *et al.*, 1993; Gibbs *et al.*, 1997; Thompson *et al.*, 2003). PCR products were separated by agarose gel electrophoresis and visualised using ethidium bromide staining. When all potyvirus assays or two out of three of the tests produced cDNA of the expected sizes, the tests were assumed to have successfully detected the virus isolate. When only one test produced cDNA of the expected size, further work was done to generate sequence data to confirm the test result and virus isolate identity.

Table 3.3 Nucleotide sequences, annealing temperatures and expected amplicon sizes of primer pairs used in each PCR assay

PCR assays	Primers	Primer Sequences 5'-3'	Tm (°C)	Expected Amplicon Size (bp)
NAD	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	55	188
	AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT		
NIb	NIb2F	GTITGYGTIGAYGAYTTYAAYAA	45	350
	NIb3R	TCIACIACIGTIGAIGGYTGNCC		
Potyvirid	Potyvirid primer 1	CACGGATCCCGGG$(T)_{17}$VGC	60	1600-2100
	Potyvirid primer 2	ACCACAGGATCCGGBAAYAAYAGYG GDCARCC		
WCIEN	CN48	TCGTGIATHGANAATGG	42	~700
	Oligo dT	$(T)_{21}$V		

N=A+C+G+T, V=A+C+G, R=A+G, W=A+T, Y=C+T. I= deoxyinosine

3.2.2 NZ MAF evaluation of NIb2F-NIb3R primer set for potyvirus detection.

In the NZ MAF laboratory, the NIb assay was tested on a total of 20 potyvirus-infected samples in two separate tests (Figure 3.1 and Figure 3.2) and compared to a previously published primer set Oligo-1n and Oligo-2n (Marie-Jeanne *et al.*, 2000). In test 1, a total of 17 potyvirus-infected samples, plus a tritimovirus control wheat streak mosaic virus (WSMV; sample 15) were tested (samples 1-17; Table 3.4). The NIb2F and NIb3R primers were able to detect 14 out of 16 potyvirus positive controls (88.2%), as well as RGMV and WSMV, but failed to detect SPVG (sample 8) and PPV (sample 17) which was isolated from Agdia PPV strip (Figure 3.1 and Table 3.4). The Oligo1n/2n was able to detect 17 out of 17 (100%) potyvirus positive controls, but failed to detect WSMV, a tritimovirus.

In test 2, the assays were applied to a total of four potyvirus-infected samples (samples 8, 18, 19 and 20), as well as WSMV (sample 15;). The NIb assay detected all five samples tested, including SPVG (different RNA extract to test 1) and WSMV, whereas the Oligo1n/2n assay detected all five potyvirus isolates and not the tritimovirus control (Figure 3.2).

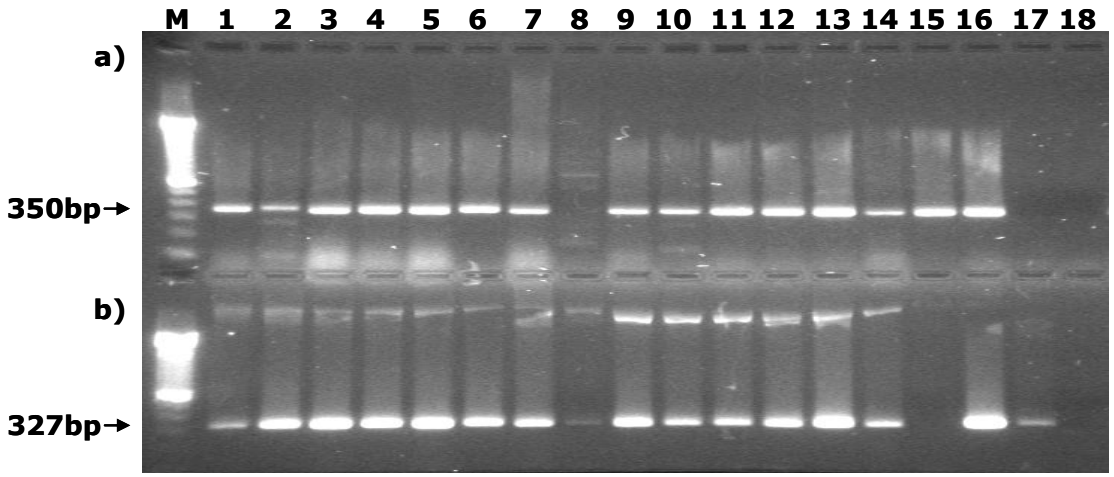

Figure 3.1 Electrophoresis of PCR products generated by a) NIb2F-3R primers (NIb assay) and b) Oligo1n/2n primers in test 1

Lane M = DNA molecular weight marker X (Roche); Lanes 1-18 are: 1) PVA; 2) IMMV+OrMV; 3) NYSV; 4) ZaMV; 5) TuMV; 6) PPV(from prunus); 7) LYSV; 8) SPVG; 9) ApVY; 10) BYMV; 11) LMV; 12) PVY; 13) RyMV; 14) WVMV; 15) WSMV; 16) ZYMV; 17) PPV (from Agdia PPV strip); 18) No template control (NTC)

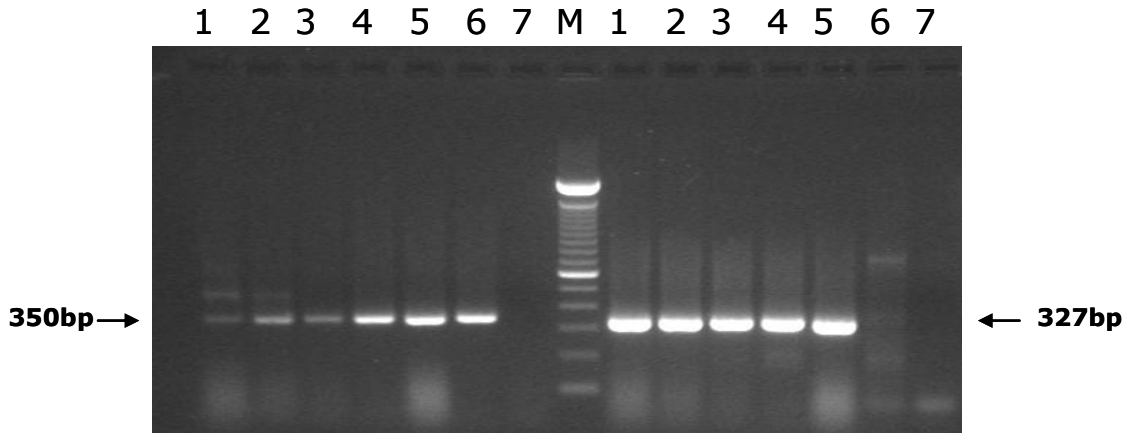

Figure 3.2 Comparison of RT-PCR results on potyvirus isolates using NIb2F-3R primers (left panel) and oligo1n/2n primers (right panel) in test 2

Lanes 1-7 are: 1) SPFMV+SPV2+SPVG (RNA extract 1); 2) SPFMV+SPV2+SPVG (RNA extract 2); 3) SPVG; 4) DsMV; 5) ClYVV; 6) WSMV and 7) NTC

Table 3.4 Names and acronyms of potyvirus positive controls used in the New Zealand MAF laboratory, their host information and the RT-PCR results of the NIb assay

	Virus Name	Acronym	Host	RT-PCR results
1	*Potato virus A*	**PVA**	*Solanum* (tamarillo)	**+**
2	*Iris mild mottle virus* *Ornithogalum mosaic virus*	**IMMV+OrMV**	*Iris*	**+**
3	*Narcissus yellow stripe virus*	**NYSV**	*Narcissus*	**+**
4	*Zantedeschia mosaic virus*	**ZaMV**	*Zantedeschia*	**+**
5	*Turnip mosaic virus*	**TuMV**	*Tropaeolum majus*	**+**
6	*Plum pox virus*	**PPV**	*Prunus*	**+**
7	*Leek yellow stripe virus*	**LYSV**	*Allium* (garlic)	**+**
8	*Sweet potato virus G*	**SPVG**	*Ipomoea* (sweet potato)	**+**
9	*Apium virus Y*	**ApVY**	*Conium* (hemlock)	**+**
10	*Bean yellow mosaic virus*	**BYMV**	*Phaseolus*	**+**
11	*Lettuce mosaic virus*	**LMV**	*Lactuca* (lettuce)	**+**
12	*Potato virus Y*	**PVY**	*N.tobacum*	**+**
13	*Ryegrass mosaic virus*	**RGMV**	*Lolium* (ryegrass)	**+**
14	*Wisteria vein mosaic virus*	**WVMV**	*Wisteria*	**+**
15	*Wheat streak mosaic virus*	**WSMV**	*Triticum* (wheat)	**+**
16	*Zucchini yellow mosaic virus*	**ZYMV**	*Cucurbita* (zucchini)	**+**
17	*Plum pox virus*	**PPV**	Agdia PPV strip	**-**

	Virus Name	Acronym	Host	RT-PCR results
18	*Sweet potato feathery mottle* *Sweet potato virus 2* *Sweet potato virus G*	**SPFMV+SPV 2+SPVG**	*Ipomoea* (sweet potato)	**+**
19	*Dasheen mosaic virus*	**DsMV**	*Amorphophallus*	**+**
20	*Clover yellow vein virus*	**ClYVV**	*Pisum*	**+**

3.2.3 Discussion

The NIb2F-3R primers detected 19 out of 20 potyvirus samples tested by the NZ MAF laboratory, representing a total of 20 potyvirus isolates, one rymovirus (sample 13, RGMV) and one tritimovirus (sample 15, WSMV). The Oligo1n/2n primers also detected all potyvirus isolates tested and the amplicons produced appear brighter when separated on agarose gels. Interestingly, the NIb2F-3R primers detected the PPV isolate in sample 6 but not the isolate isolated from the Agdia PPV strip (sample 17). This could be due to the quality of RNA isolated from the strip and/or the inhibitors present in the isolated RNA.

The NIb primers detected the sample containing a mixed infection of SPFMV, SPV2 and SPVG, as well as a single infection of SPVG, albeit with less amplicons when viewed on ethidium-bromide stained agarose gels. The weaker gel banding patterns produced suggest that the primers might not be annealing efficiently to the targets. This could be due to a lack of SPFMV, SPV2 and SPVG genome sequences in the initial design process of the NIb primers, as none of these species have been fully sequenced. The NIb2F-3R primers could be improved by using partial sequences in the NIb region, to allow for possible new variations that exist in species not yet fully sequenced.

3.3 Evaluation of universal primers for nepovirus detection

Two sets of universal primers, NepoA-F/R and NepoB-F/R, designed to detect group A and B of the nepovirus genus respectively (Two separate tests were conducted using a total of 14 samples, 10 of which were isolates of *Grapevine fanleaf virus* (GFLV); two were isolates of *Tomato ringspot virus* (TRSV), both belong to nepovirus group A and two samples suspected of nepovirus infections. Samples 1-12 were tested in the first test using both the NepoA and NepoB universal primers in a 2 step RT-PCR assay following the published protocol (Wei *et al.*, 2008). Both assays did not produce any positive identification. The NepoA generic assay produced a positive result for sample 13 whereas the NepoB generic assay detected sample 14 (Table 3.6). Cloning and sequencing are required to identity these two as yet unidentified nepovirus isolates.

Table 3.5), were validated and published by our New Zealand colleagues (Wei *et al.*, 2008). As part of this collaborative project, the Victorian DPI laboratory also validated these published primers and a summary of the validation follows.

Two separate tests were conducted using a total of 14 samples, 10 of which were isolates of *Grapevine fanleaf virus* (GFLV); two were isolates of *Tomato ringspot virus* (TRSV), both belong to nepovirus group A and two samples suspected of nepovirus infections. Samples 1-12 were tested in the first test using both the NepoA and NepoB universal primers in a 2 step RT-PCR assay following the published protocol (Wei *et al.*, 2008). Both assays did not produce any positive identification. The NepoA generic assay produced a positive result for sample 13 whereas the NepoB generic assay detected sample 14 (Table 3.6). Cloning and sequencing are required to identity these two as yet unidentified nepovirus isolates.

Table 3.5 Nucleotide sequences and expected amplicon sizes of primer pairs used in nepovirus-specific RT-PCR assays

RT-PCR assays	Primers	Primer Sequences 5'-3'	Expected Amplicon Size (bp)
Nad	AtropaNad2.1a	GGACTCCTGACGTATACGAAGGATC	188
	AtropaNad2.2b	AGCAATGAGATTCCCCAATATCAT	
NepoA	F (forward)	ACDTCWGARGGITAYCC	~340
	R (reverse)	RATDCCYACYTGRCWIGGCA	
NepoB	F (forward)	TCTGGITTTGCYTTRACRGT	~250
	R (reverse)	CTTRTCACTVCCATCRGTAA	

In test 2, minor adjustments were made to the reaction setup and cDNA synthesis step of the 2-step RT-PCR in an attempt to improve the sensitivity of the universal primers. The SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used following the manufacturer's recommendations. Briefly, the cDNA synthesis reaction consisted of: 4 µL of 5x first strand buffer, 0.5 µL of random hexamers (50 ng/µL), RNasin (40U/µL), 4 µL of total RNA extract and made up to a volume of 16 µL with DNase/RNase free H_2O. The mixture was incubated at 95^oC for 10mins before holding at room temperature for 15 mins. A cocktail containing 0.5 µL of Superscript III reverse transcriptase (200U/µL), 0.1 µL of RNasin (40U/µL), 2 µL of 0.1 M DTT, 1 µL of 10 mM dNTPs and 0.4 µL of H_2O were added to the cDNA synthesis reaction and the mixture was incubated at 42^oC for 1 hour.

Samples 13 and 14 were tested using the improved cDNA synthesis and PCR reaction setups (Platinum taq and dNTP bundle kit, Invitrogen; Table 3.7) and the published PCR cycling conditions (Wei *et al.*, 2008). The NepoA generic assay produced a positive result for sample 13 whereas the NepoB generic assay detected sample 14. Cloning and sequencing work is required to unambiguously confirm the identity of these two unknown nepoviruses.

Table 3.6 Names and acronyms of nepovirus positive controls, their host information and the RT-PCR results of the nepo-specific RT-PCR assays

Sample Number	Virus Name	Acronym	Host	RT-PCR results	
				NepoA	NepoB
1	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
2	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
3	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
4	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
5	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
6	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
7	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
8	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
9	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
10	*Grapevine fanleaf virus*	**GFV**	*Vitis vinifera*	-	-
11	*Tomato ringspot virus*	**TRSV**	*N/A*	-	-
12	*Tomato ringspot virus*	**TRSV**	*N/A*	-	-
13	*Unknown*	-	*Prunus*	+	-
14	*Unknown*	-	*Blueberry*	-	+

Table 3.7 Improved PCR setup for nepovirus-specific generic assays

Reagents	1x (25 µL)
10x PCR buffer	2.5
50 mM $MgCl_2$	1
dNTPs (10 mM)	0.5
Primer F (10 µM)	3.75
Primer R (10 µM)	3.75
Platinum taq	0.25
H_2O	10.75
cDNA	2.5

3.4 Diagnostic assay development for viruses in the genera Furovirus, Hordeivirus, Rymovirus and Tritimovirus

3.4.1 Introduction

Diagnostic assays based on DNA detection technology have been designed to detect a group of closely related cereal viruses through the use of universal primers. A novel approach of designing universal primers has been thoroughly explored by Zheng et al (2008) using members of the genus Potyvirus as a model. The same approach can be utilised in the development of generic assays for viruses that pose a threat to winter cereals, namely viruses from the genera *Furovirus, Hordeivirus, Rymovirus* and *Tritimovirus*, provided that sufficient sequence data is available. The aim of this project is to analyse the sequence data currently available for universal primer design and develop generic assays for the detection of the cereal viruses. As part of Phase II of the PEQ project it is planned that the primer sets that are recommended for detection of these four plant virus genera will be further validated in the New Zealand MAF laboratory,

3.4.1.1 Furoviruses

Furoviruses is a group of fungus-transmitted rod-shaped plant virus that infect graminaceous crop plants such as wheat, barley, rye, oat and sorghum, causing yellow to green mosaic on leaves and stunting of plants. *Soil-borne wheat mosaic virus* (SBWMV) is the type species and other members of the group are *Chinese wheat mosaic virus* (CWMV), *Oat golden stripe virus* (OGSV), *Soil-borne cereal mosaic virus* (SBCMV) and *Sorghum chlorotic spot virus* (SgCSV) (Table 3.8) (Fauquet *et al.*, 2005). There are no tentative species reported in the genus.

With the exception of SgCSV, all other members of the *Furovirus* can cause a severe yield loss of up to 80% in grain production, depending on variety, climatic conditions and time of sowing (Clover et al,. 2001 book p583). In Italy, furovirus infections are estimated to cause about 48% grain yield loss in wheat (Vallega et al.,1999 from (Lapierre *et al.*, 2004). A brief summary outlining the disease and basic biological properties, including their vectors/transmission modes, natural hosts, geographical distribution and detection methods of the five confirmed members of the *Furovirus* is as follows (Table 3.8).

Table 3.8 Summary of the natural vectors, hosts and geographical distribution, disease symptoms and detection methods of furoviruses

Confirmed species	Acronym	Natural Vector/s	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Chinese wheat mosaic virus	CWMV	*Polymyxa graminis*	Wheat	China	Light chlorotic streaking on the young leaves and bright yellow cholorotic streaking on older leaves. The infect plants are severely stunted, wilt and later die. CWMV usually occurs in mixed infections with *Wheat yellow mosaic virus* (WYMV) and causes commonly 10-30%, but sometimes up to 80%, loss of grain yield.	Serological methods, i.e. ELISA, ISEM and nucleic acid based methods, i.e. RT-PCR and Northern blot (p568)
Oat golden stripe virus	OGSV	Believed to be *Polymyxa graminis* Ledingham	Oats	UK, Irish Republic, France and the south-east USA	Plants may have symptomless infection or show bright yellow, elongated streaks on leaves. OGSV usually occurs in mixed infections with *Oat mosaic bymovirus* (OMV) and can cause up to 60% yield loss. (Lapierre *et al.*, 2004)	Manual inoculation to *Nicotiana clevelandii* Gray or *Chenopodium amaranticolor* Coste & Reyn. and serological methods
Soil-borne cereal mosaic virus	SBCMV	*Polymyxa graminis* and seed-borne transmission (Budge *et al.*, 2008)	Rye (*Secale cereale* L.), wheat species (*Triticum aestivum* L., *T. durum* Desf., *T. turgidum* L.) and triticale	Germany, Poland, Demark, UK, Italy, France, possibly North America and Africa	Symptoms of SBCMV infection vary greatly amongst different hosts and susceptibility of the cultivar and season. On wheat, triticale and rye, SBCMV causes streaking rather than mottling symptoms. Leaf streaks may range in colour from mild green to yellow. Young leaves appear mottled and develop pale flecks that cover both the leaf lamina and sheath. 50% reduction in tiller length. SBCMV often occurs in mixed infections with *Wheat spindle streak mosaic bymovirus* (WSSMV). Yield loss caused by SBCMV is difficult to determine due to misdiagnosis and the lack of discrimination between SBCMV and SBWMV.	Visual assessment of infected plants, but often misdiagnosed. Manual inoculation to indicator plants, serological methods (i.e. ELISA, tissue print immunoassay, ISEM and Western blots) or molecular methods (i.e. IC-RT-PCR and RT-PCR) (Lapierre *et al.*, 2004)

Confirmed species	Acronym	Natural Vector/s	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Soil-borne wheat mosaic virus	SBWMV	*Polymyxa graminis* Ledingham and possibly via seed transmission (Lebas *et al.*, 2009)	*Triticum* species, *Hordeum vulgare* L., *Secale cereale* L., *Bromus commutatus.* Schrad.	Central USA, China, Japan, France, Italy, Argentina, Brazil and New Zealand	Symptoms of SBWMV include green or yellow mosaic, severely stunted plants (rosetting disease) with reduced kernel weight. Plants with mosaic disease are often more stunted than those with a green mosaic disease. In central USA, SBWMV can cause up to 80% yield loss in winter wheat fields. In China yield losses were reported to be 10-30% and as high as 70%. Mixed infections of SBWMV and WSMV induce symptoms that include mosaic, yellowing, bronzing, reduced plant height and tillering. Mixed infections also cause a disease in wheat plants that is more severe than the disease caused by each virus alone (Lapierre *et al.*, 2004).	Visual assessment of wheat, ELISA analysis with monoclonal antibodies, RT-PCR assays
Sorghum chlorotic spot virus	SgCSV	Believed to be *Polymyxa graminis* Ledingham	*Sorghum bicolour* (L.) Moench	USA	Strikingly prominent elongated, elliptical green islands and yellow mosaics, chlorotic spots. This virus has only been isolated once in from naturally infected sorghum and is not known to cause a disease of economic consequence (Lapierre *et al.*, 2004).	Manual inoculation to *Nicotiana clevelandii* or *Chenopodium* species or ELISA

Genome organisation of furoviruses

Virus species from the genus *Furovirus* have a bipartite genome, RNA1 and RNA2. Both RNA components are single stranded, positive sense molecules, with RNA1 being the larger of the two. RNA1 encodes a replication protein, with a readthrough protein of RNA-dependent RNA polymerase (RdRp). RNA2 encodes the coat protein (CP) and the readthrough region of the CP is probably required for particle assembly and for transmission by the plasmodiophorid vector.

There is a further (3'-proximal) gene on each of the RNAs and these are translated from shorter RNA molecules transcribed from the 3'-end of the genomic RNA ('subgenomic' mRNAs). The 3'-proximal gene on RNA1 encodes a cell-to-cell movement protein (MP) that enables the virus to move between adjacent plant cells via the plasmodesmata while the function of the product from RNA2 is uncertain but may involve suppression of the host plant defence reaction (Lapierre *et al.*, 2004).

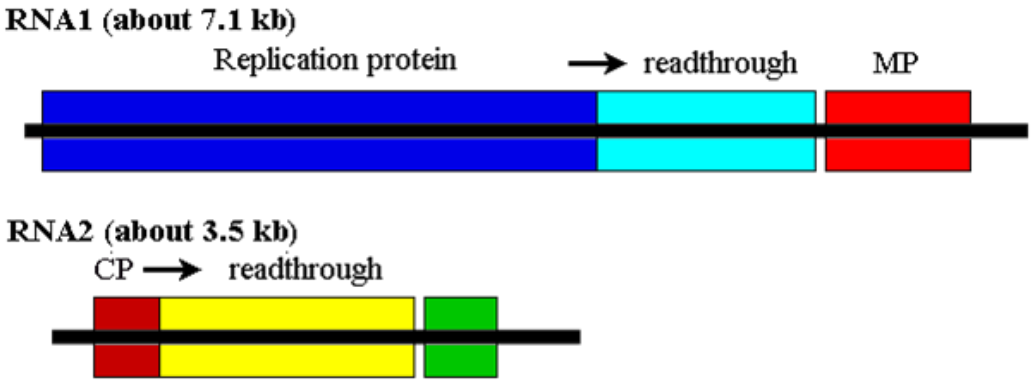

Figure 3.3 Schematic genome organisation of a typical furovirus

3.4.1.2 Hordeiviruses

The genus *Hordeivirus* is not assigned to any virus family and to date, it consists of four confirmed species and no tentative species. The type species of *Hordeivirus* is *Barley stripe mosaic virus* (BSMV) and the other three species are *Poa semilatent virus* (PSLV), *Lychnis ringspot virus* (LRSV) and *Anthoxanthum latent blanching virus* (ALBV).

Infection symptoms caused by hordeiviruses include a very mild mosaic, yellow or white chlorotic striping and sometimes necrosis. The viruses are mechanically and seed/pollen transmitted, with no known virus vectors.

A brief summary outlining the disease and basic biological properties, including their vectors/transmission modes, natural hosts, geographical distribution and detection methods of the five confirmed members of the *Hordeivirus* is as follows (Table 3.9).

Table 3.9 Summary of the natural vectors, hosts and geographical distribution, disease symptoms and detection methods of hordeiviruses

Confirmed species	Acronym	Transmission	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Anthoxanthum latent blanching virus	ALBV	Mechanical, pollen and/or seed transmission	*Anthoxanthum odoratum* (sweet vernal grass)	Wales and England	Infected *A. odoratum* are mostly symptomless, but can develop small, distinct, pale yellow patches. A few develop irregularly shaped white patches of various sizes, which rarely spread across the leaf blade, followed by leaf wilt and death (Lapierre *et al.*, 2004) pp 730)	Mechanical inoculation to *Chenopodium* species, *A. odoratum, L ovatus* and/or serological methods (i.e. ELISA, ISEM)
Barley stripe mosaic virus	BSMV	Seed, pollen or direct leaf contact	Barley and occasionally wheat and wild oats	World-wide	Very mild mosaic, yellow or white chlorotic striping and sometimes necrosis. Infected plants can be stunted and maybe sterile or produce shrivelled seeds. Up to 60% yield losses have been reported in barley. (Lapierre *et al.*, 2004) pp 457)	Manual inoculation to *Chenopodium* species, electron microscopy, serological methods (i.e. ELISA) and nucleic acid hybridization techniques
Lychnis ringspot virus	LRSV	Mechanical inoculation and seeds; probably transmitted by grafting	*Lychnis divaricata, L. chalcedonica, L. coronaria, L. haageana*	Hungary	Systemic ringspots, mosaic and necrosis	Biological assays with susceptible hosts and ELISA (ICTV DB Description. http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm)
Poa semilatent virus	PSLV	Mechanical transmission	*Elymus trachycaulus* and *P. palustris.*	Canada	Severe isolates cause blight and rapid death of plants while mild isolates cause chlorotic mottling and limited necrosis. Yield loss is not extensive.	Immunological tests and experimental host range analysis (Lapierre *et al.*, 2004) pp 783)

Genome organization

Virus species from the genus *Hordeivirus* have a tripartite genome, all of which are positive sense ssRNAs ranging from 2.6-3.9kb in size (Lapierre *et al.*, 2004). There are a total of seven reading frames. RNAα is the largest subgenomic RNA molecule, and encodes a single protein that includes a subunit of the RNA dependent RNA-polymerase (RDRP). RNA beta (RNAβ) is the second largest subgenomic component, containing four major open reading frames (ORFs), which encodes for the coat protein (CP) and three triple gene block (TGB) proteins. RNAγ is the smallest ssRNA component, which encodes two proteins, including the polymerase subunit of the RdRp (Figure 3.4).

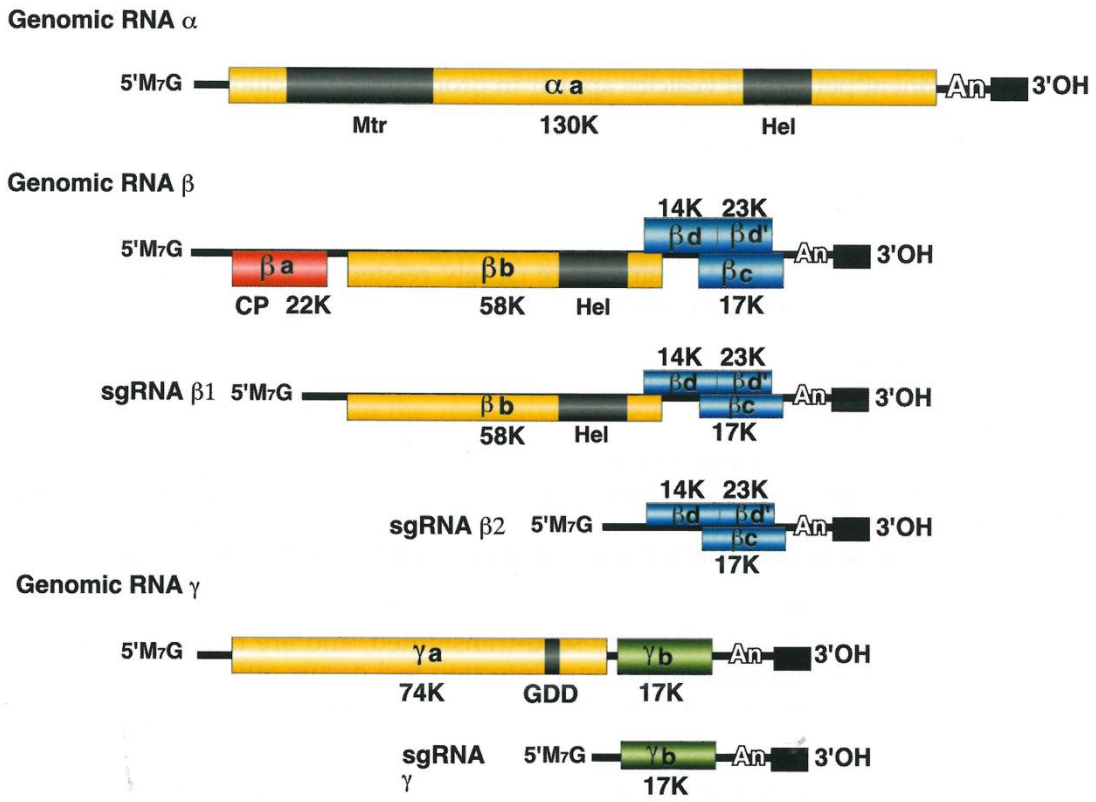

Figure 3.4 A schematic representation of the hordeivirus genome organisation (example given here is of Barley stripe mosaic virus**)**

The open rectangle and the solid filled rectangles represent the ORFs and the 3′-terminal tRNA-like structure respectively.(Badge *et al.*, 1996)

3.4.1.3 Rymovirus

The genus *Rymovirus* belongs to the family *Potyviridae* and the name of the genus derived from its type species *Ryegrass mosaic virus* (RGMV). There are two other confirmed species in the *Rymovirus* genus, *Agropyrum mosaic virus* and *Hordeum mosaic virus* (Table 3.10), all of which contain a linear, single molecule of positive sense single-stranded RNA that is approximately 9.5kb in length (Figure 3.5). The taxonomic of status of species in the genus is currently in flux. Although *Oat necrotic mottle virus* (ONMV) is currently listed as a definitive member of *Rymovirus* (Fauquet et al., 2005), serological and nucleotide sequence data from various studies (Rabenstein *et al.*, 2002) suggest that ONMV should be reclassified as a tritimovirus. Similarly, results from both serological and

molecular analysis suggest that the tentative rymovirus species Spartina mottle virus (SpMV) may constitute a new genus as it has no close relationship with any definitive member of the genus *Rymovirus* (Gotz *et al.*, 2002). The name Sparmovirus is proposed for the new genus, for which SpMV would represent the type member. In this study, both ONMV and SpMV were not considered to be members of the *Rymovirus* and as such, sequence data from both species were not used for the purpose of rymovirus-specific primer design.

Natural host range for rymoviruses are restricted to species of the *Poaceae* and transmitted by mite vectors. General symptoms of a rymovirus infection include systemic cholorotic and/or necrotic streaking and mosaic of varying severity on hosts. A brief summary outlining the disease and basic biological properties, including their vectors/transmission modes, natural hosts, geographical distribution and detection methods of the three confirmed members of the *Rymovirus* is as follows (Table 3.10).

Genome organisation of rymoviruses

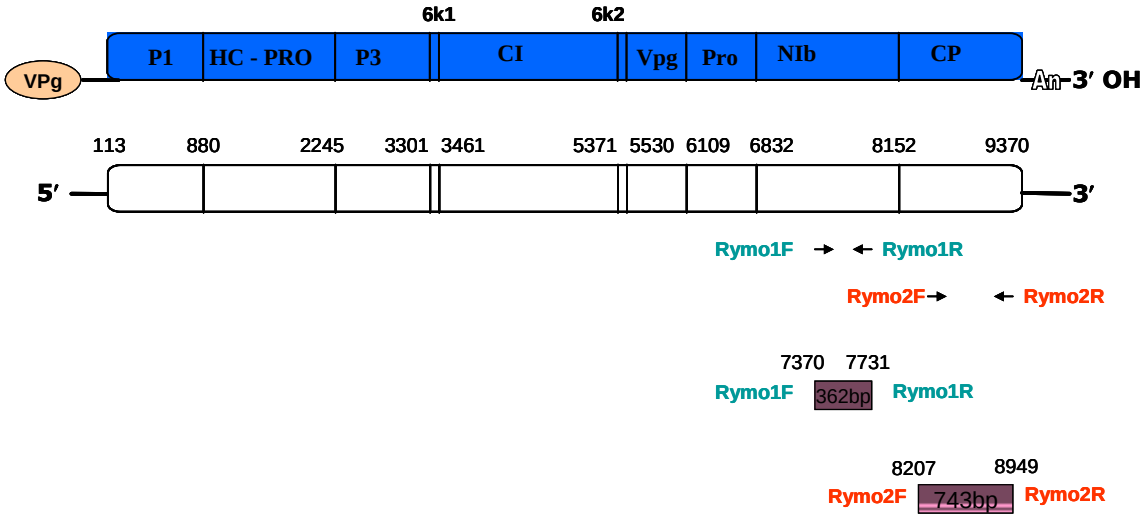

Figure 3.5 Schematic representation of a rymovirus genome and locations of universal primers designed for rymovirus detection

Table 3.10 Summary of the natural vectors, hosts and geographical distribution, disease symptoms and detection methods of rymoviruses

Confirmed species	Acronym	Transmission	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Agropyrum mosaic virus	AgMV	Believed to be Abacarus hystrix (Nalepa)	*Agropyron* species or wheat, *Aristida* sp., *Bothriochloa laguroides* (DC.) Herter, *Elymus smithii* (Rydb.) Gould and *Sorghastrum nutans* Nash.	USA, southern Canada, Finland, Germany, Great Britain and Hungary	Some strains can cause severe stunting and yield losses. Effects of AgMV alone on wheat are not known, but dual infection with WSMV was reported to be up to 85% yield loss in an Oklahoma farm. ((Lapierre *et al.*, 2004) pp 726)	Serological methods, nucleic acid hybridization and reverse transcription-polymerase chain reaction. Variants can be determined via complete genome sequencing
Hordeum mosaic virus	HoMV	Unknown and can be mechanically transmitted	Barley, wild perennial grasses *Agropyron trachycaulum* (Link), *Agrohordeum (Elymus) macounii* (Vasey) Lepage and *Hordeum jubatum* L.	Canada	Mottle on wheat, rye, oat and other grasses. Agronomical importance of HoMV is minimal. ((Lapierre *et al.*, 2004) pp474)	Mechanical transmission to susceptible hosts, serological methods (i.e. DAS-ELISA, ELISA, Western blotting or immunoelectron microscopy)

Confirmed species	Acronym	Transmission	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Ryegrass mosaic virus	RGMV	Eriophyid mite *Abacarus hystrix* (Nalepa). Argenine stem weevil (*Listronotus bonariensis* Kuschel) (*Coleoptera*) is a casual vector	Festucoid species within the family *Poaceae. Lolium perenne* L., *L. multiflorum* Lam. And *Dactylis glomerata* L.	England, Wales, Scotland, Holland, Washing state USA, Canada, parts of Europe, Australia, New Zealand and South Africa	Pale green to yellow mosaic or streaking on the leaf lamina of *Lolium* species and in severe cases, the mosaic is accompanied by a brown discoloration which is often followed by necrosis. Slows plant growth and lowers secondary tiller production. It is considered, after barley yellow dwarf virus, the most serious and widespread virus affecting fodder grasses. Infections with virulent strains may decrease the yield of Italian ryegrass up to 50%.	Manual inoculation to suceptible hosts, serological methods (i.e. ELISA) with monoclonal antibodies or immunocapture RT-PCR and RT-PCR ((Lapierre *et al.*, 2004) pp 788)

3.4.1.4 Tritimovirus

The genus *Tritimovirus* has two definitive members, *Wheat streak mosaic virus* (WSMV) and *Brome streak mosaic virus* (BStMV). A possible third member, *Oat necrotic mosaic virus* (ONMV) is still officially classified as a rymovirus, but recent serological and molecular analyses indicate that it should be classified as a tritimovirus instead.

The natural host range of tritimoviruses is restricted to species of the *Poaceae,* vectored by eriophyid mite *Aceria tosichella* Keifer, and transmissable by seed. Symptoms of tritimovirus infection include systemic chlorotic and/or necrotic streaking of leaves and on older leaves, a mosaic of discontinuous yellow-green to yellow or brown streaks of varying severity.

A brief summary outlining the disease and basic biological properties, including their vectors/transmission modes, natural hosts, geographical distribution and detection methods of the two confirmed members and the possible third member of the *Tritimovirus* is as follows (Table 3.11).

Table 3.11 Summary of the natural vectors, hosts and geographical distribution, disease symptoms and detection methods of tritimoviruses

Confirmed species	Acronym	Transmission	Natural host/s	Geographical distribution	Disease symptoms	Detection methods
Brome streak mosaic virus	BStMV	Mechanical inoculation and vectored by the mite *Aceria tulipae* Keifer	Some species in the genera *Avena, Bromus, Hordeum, Triticum* and *Vulpia*, including cereals wheat, barley and oat.	Wild grasses in Jugoslavia and Germany; cereals wheat and barley in France	Chlorotic streaks and stripes on leaves of infected plants, strong chlorosis appears with yellow streaks and spots The agronomical importance of BStMV is not know, although the potential can be great due to its transmutability onto wheat, barley and oat	Mechanical inoculation to host species, especially barley, serological methods (i.e. ELISA) or molecular tests (i.e. RT-PCR)
Wheat streak mosaic virus	WSMV	seed transmission in maize and wheat; vectored by the wheat curl mite *Aceria tritici* (Shevtchenko) and readily mechanically transmissible	Wheat, maize and many wild grasses	USA, Mideast, Central Europe, France, Mexico and Australia	Mosaic of discontinuous yellow-green to yellow streaks on leaves; leaves with their edges rolled in toward the midrib. Infected plants have less erect tillers than healthy plants and are stunted with rosette appearances. Sterile heads often develop, resulting in considerable wheat yield losses. Yield losses can be as high as 100% depending on environmental conditions	Manual inoculation to seedlings of *Triticum aestivum* L., serological methods (i.e. ELISA) and RT-PCR
Oat necrotic mottle virus	ONMV	No known vector, can be mechanically transmitted		Canada, Germany	Systemic mosaic and fine chlorotic streaking on *P. pratensis;* necrosis and green mosaic symptoms on oat Agronomical importance of ONMV is currently negligible, but it may cause serious problems since all lines of oats tested are susceptible to the virus	Host range studies, serological methods (i.e. DAS-ELISA, Western blotting or immunoelectron microscopy) or RT-PCR

3.4.2 Materials and Methods

3.4.2.1 Import permits and positive virus controls

An import permit (IP08004124) for nucleic acids extracted from furoviruses, hordeiviruses and rymoviruses was obtained through AQIS as part of this project. The procurement of this permit was extremely important for the success of the project as positive virus controls are pivotal in validation of any new or established diagnostic assays.

A total of 16 virus isolates was imported from three distinct geographical locations: Germany, New Zealand (NZ) and United Kingdom (UK). Virus isolates imported from Germany and New Zealand came in the form of extracted nucleic acids suspended in ethanol, whereas virus isolates imported through UK were transported as plant sap spotted onto FTA (Flinders Technology Associates) cards (Whatman, UK).

3.4.2.2 Group-specific universal primer design

Alignment was generated using sequences from the most comprehensive dataset using the mltiple sequence alignment program MUSCLE (Edgar, 2004). Conserved sites within the alignment were identified using the nucleotide conserved site finder (NCSF) program (Zheng *et al.*, 2008b) using five different measures: average nucleotide variants, entropy, minimum redundancy, minimum variants and maximum count with the site length parameter set to 20. Conserved sites were also identified by eye, where the site must be equal or greater than 17bp in length, with two absolute conserved positions followed by a wobble base position. The top five sites chosen by each measure, along with sites that were identified by eye were analysed for their rate of consensus decay using methods previously published (Zheng *et al.*, 2008b). The resultant 'average nucleotide (N) score' of the sites were used to rank the sites. The best ranked sites were assessed for their suitability as primer targets based on their sequence information, such as the possibility of self-hybridisation and primer dimer formation using the program OligoAnalyzer 1.2 (Gene-Link software) and OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html).

Suitable sites must yield primers with no secondary RNA structure (i.e. internal loops) and have a minimum chance of self-hybridisation. Sites with sequences that might self-hybridise at the 3′ end as predicated by the program OligoCalc were deemed unsuitable for universal primer design. Sites were also selected for their proximity to each other (<1kb apart) to allow for efficient amplification of target sequences; sites that were located more than 1kb apart were not considered for universal primer design.

Deoxyinosine bases (dI) were incorporated into the universal primers to reduce the degeneracy of the primers as required, usually at positions of greater than 3-fold degeneracy. As a general rule, no more than 4 dIs were incorporated into any primer and all dIs were placed more than positions away from the 3′ end of the primer.

3.4.2.3 RNA isolation

Previously tested virus isolates from the genera *Furovirus, Hordeivirus, Rymovirus* and *Tritimovirus* were used as positive virus controls for generic assay validation. With the exception of the Australian isolate of *Wheat streak mosaic virus* (WSMV; tritimovirus), all other virus isolates were imported either suspended in alcohol or on FTA cards (Whatman, UK).

RNA extraction from leaf tissue

RNA from the Australia isolate of *Wheat streak mosaic virus* was extracted using a standard RNA extraction method. Total RNA was extracted from approximately 0.1 g of freeze-dried or 0.2 g of fresh plant tissues using the RNeasy Plant Mini Kit (Qiagen) and a modified lysis buffer (Mackenzie et al., 1997). RNA extracts were then eluted in a final volume of 50 µL.

Re-isolation of RNA suspended in ethanol

The extracted nucleic acid samples suspended in ethanol are re-isolated via a standard ethanol precipitation process whereby the samples were subjected to a centrifugation at a speed of 10,000 xg at room temperature for 30 minutes before the supernatant was discarded. The resulting pellet (usually invisible) was washed with 70% ethanol twice before re-centrifugation for 10 minutes at room temperature. The samples were allowed to air dry before suspension in 30 µL of RNase/Dnase-free water and stored at -20^{o}C until use.

RNA isolation from FTA cards

Viral RNA was isolated from the plant sap spotted on FTA cards following the manufacturer's protocol (Whatman, UK). Briefly, four 6 mm discs were excised from the sample spot using a Harris Uni-Core puncher and placed in a sterile 1.5 mL tube. One-hundred uL of RNA processing buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 800U/mL RnaseOut (Invitrogen), 2 mM DTT) was added to the sample in the tube. The mixture was briefly agitated before and after the sample was incubated at room temperature for 15 minutes. The eluted RNA was further concentrated using a standard ethanol precipitation. Specifically, 1/10 volume of 3 M sodium acetate (pH 5.2) and an equal volume of ice-cold 100% isopropanol were added to the elution before an incubation at -20^{o}C for one hour. The mixture was then centrifuged at 10,000 xg for 30 minutes before the supernatant was discarded. The resultant pellet was washed with 250 µL of ice cold 75% ethanol and then centrifuged at 10,000 xg for five minutes at room temperature. The supernatant was discarded and the sample allowed to air dry at room temperature. The pellet was then suspended in 50 µL of TE^{-1} (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer and stored at -20^{o}C until use.

3.4.2.4 RT-PCR and cycling conditions

All nucleic acid extracts were tested by RT-PCR for the presence of viral RNA using each of their respective universal primer sets, i.e. the Furo1F-1R, Furo1F-2R and Furo2F-2R universal assays were applied to all furovirus isolates. All samples were also tested for the presence of amplifiable RNA using the house-keeping gene NAD primer pair Nad2.1a and Nad2.2b. One-step RT-PCR was performed using Superscript™ III Platinum Taq (Invitrogen, USA) in a 25 µL volume reaction as per the manufacturer's instructions, with 1µL of isolated RNA. All universal primers were used at a final concentration of 0.5 µM. A reverse transcription step was done at 48^{o}C for 45 minutes and terminated at 94^{o}C for 2 minutes. PCR amplification immediately followed, with 35 cycles at: 95^{o}C for 30 seconds, variable annealing temperature (assay dependent) for 30 seconds and 72^{o}C for 20-40 seconds dependent on the assay; a final extension of five minutes was done at 72^{o}C and the reactions were then held at room temperature. PCR products were separated by agarose gel electrophoresis and visualised using ethidium bromide staining. When the assay produced amplicons of the expected sizes, the assay was assumed to have successfully detected the virus isolate.

3.4.3 Results

3.4.3.1 Furovirus

Furovirus datasets

Nucleotide sequences from all members within the genus *Furovirus* were retrieved from the public nucleotide sequence database Genbank. All sequences were dated manually using their deposition dates in GenBank before being separated into full-length and partial sequence datasets, as only full-length sequences were considered for the purpose of universal primer design. The full-length sequences were then sorted into subsets of sequences, 'RNA1' and 'RNA2', where each subset contained nucleotide sequences from the RNA1 and RNA2 of the furovirus genomes respectively.

The number of distinct species represented by the full-length sequences from each dataset was determined. Only the most comprehensive dataset either with a greater number of full-length sequences and/or a greater number of conserved sites was used for the purpose of universal primer design.

Identification of conserved sites in furovirus genomes

A total of thirteen full-length sequences from furovirus RNA1 were available in GenBank as of June 2009, representing all five confirmed species from the genus. The RNA1 sequence dataset is more comprehensive than the RNA2 sequence dataset, which consisted of only five full-length sequences representing four out of the five confirmed furovirus species (Table 3.12). For the purpose of designing universal primers, the full-length sequences of furovirus subgenomic RNA1 were used.

Table 3.12 Names and acronym of confirmed species in the genus Furovirus**, full-length sequences and available positive virus controls**

Confirmed species	Acronym	Full-length sequences available		Positive controls available
		RNA1	RNA2	
Chinese wheat mosaic virus	CWMV	2	1	0
Oat golden stripe virus	OGSV	1	1	1
Soil-borne cereal mosaic virus	SBCMV	5	1	3
Soil-borne wheat mosaic virus	SBWMV	4	2	3
Sorghum chlorotic spot virus	SgCSV	1	0	0

An alignment of the furovirus RNA1 full-length sequences was created and a total of 26 unique conserved sites were identified. The conserved sites were ranked based on their consensus decay rate over the past 25 years (1993- June 2009) (Table 3.13). An analysis of the top 10 sites revealed that only the top two ranked sites were suitable as target sites for furovirus-specific universal primer design. A 16th ranked site (nucleotide positions 4809-4828) was also chosen for primer design due to its proximity to the top two ranked sites (Figure 3.6,Table 3.13).

Table 3.13 The rank, nucleotide positions, sequence contents, identification method and N scores of conserved sites within the furovirus RNA1 ful-length sequences

rank	nucleotide positions[a]	nucleotide sequence of sites (5´ - 3´)	Identified by	N scores[b]
1	4179-4198[c]	AAATGACGGTTTGGGTCGAA	NCSF[d]	0.00
2	4317-4333[c]	TTCGACGGGTAYGARGT	Eye	0.12
3	7283-7304	AAGAGGGGTKCRAHTCCCCCCC	Eye	0.18
4	465-484	GAYATTGGKGGYAAYTGGGC	NCSF	0.20
4	7271-7290	GACACAGCWRYYAAGAGGGG	NCSF	0.20
4	7283-7302	AAGAGGGGTKCRAHTCCCCC	NCSF	0.20
5	4314-4333	YWYTTCGACGGGTAYGARGT	NCSF	0.25
5	4845-4864	AYGAAGGARATMAAYGARMG	NCSF	0.25
5	4980-4999	GAYTTYTCRAAATTYGAYAA	NCSF	0.25
6	7206-7225	GATGTRATTKWMRAAARATC	NCSF	0.30
6	4317-4336	TTCGACGGGTAYGARGTNKC	Eye	0.30
6	5079-5101	TGGGARAARTCKCARTSYCRGAC	Eye	0.30
7	777-802	ATKGCYATHCAYAGYATWTAYGAYAT	Eye	0.35
7	3546-3562	GTKTAYTTYTGYGCBGA	Eye	0.35
8	4971-5010	TTYGARATHGAYTTYTCRAAATTYGAYAART CVAARACDT	Eye	0.38
9	1149-1171	GAYGARTCDTTYAARTGYATHCC	Eye	0.39
10	7204-7223	YWGATGTRATTKWMRAAARA	NCSF	0.40
11	4071-4090	TCDTTRGTBTAYTAYACBGC	Eye	0.45
12	3588-3616	TGYCARGGWGAYTCNCARC	Eye	0.48
13	5133-5200	GCNTAYTTGYTDTAYCARCARAARTCNGGH AAYTGYGAYACHTAYGGHTCDAACACNTG	Eye	0.50

		GTCBGCNGC		
14	3294-3328	GAYGGHGTHCCNGGHTGYGGHAARTCBAC BTGGAT	Eye	0.51
15	5484-5506	GARATYTTYGTNTCYRTBGGDGA	Eye	0.52
16	2877-2896	CRHTGYAAGATRYHTCWGA	NCSF	0.55
16	4809-4828[c]	CCNGAYAARATHGTNAAYGC	Eye	0.55
17	228-247	TGYGANCABRTKMRNGRKMM	NCSF	0.85
18	74-93	NRVMVVSNYNYKBBDNNRMD	NCSF	1.85

[a] Nucleotide positions given are in reference to an alignment of 15 sequences from furovirus subgenomic RNA1

[b] Average nucleotide (N) scores of conserved sites within the RNA1 genomes of furoviruses

[c] Sites chosen for furovirus-specific universal primer design

[d] Nucleotide conserved sequence finder (NCSF)

Furovirus-specific universal primer designs

Four universal primers were designed for the detection of furoviruses (Table 3.14). Universal primers are inevitably degenerate in nature in order to accommodate for natural variances that occur in a set of sequences and highly degenerate primers can lower the sensitivity of the diagnostic assay. To minimise the degeneracy of these primers, a DNA analogue, deoxyinosine (dI) bases were incorporated into the degenerate positions within the universal primers as recommended by (Zheng *et al.*, 2008a). Consequently, the degeneracy of all primers designed and developed in this project is kept at or below 12-fold (Table 3.14)

Table 3.14 The nucleotide positions, sequences, senses and rank of targeted sites of group-specific universal primers for furoviruses, hordeiviruses, rymoviruses and tritimoviruses

Primer names	Nucleotide Positions	Primer sequence (5˙- 3˙)	Sense of primer	Target site rank
Furo1F	4059-4078[a]	AAATGACGGTTTGGGTCGAA	Sense	1
Furo1R	4197-4213[a]	ACYTCRTACCCGTCGAA	Anti-sense	2
Furo2F	4197-4213[a]	TTCGACGGGTAYGARGT	Sense	2
Furo2R	4689-4708[a]	GCITTIACIATYTTITCNGG	Anti-sense	16
Hordei1F	1794-1816[b]	TAIACACTIGCYGAIAGYGCKGAA	Sense	5
Hordei1R	2383-2407[b]	GIACIATIGGCCAATACTTATTNGG	Anti-sense	3
Hordei2R	2487-2506[b]	CCICCGTTIGCRAAYTTGTG	Anti-sense	4
Rymo1F	7370-7389[c]	AAGGTIGAAGCRAAYAAAAC	Sense	2
Rymo1R	7722-7731[c]	GAICCATCWGGWGTTGCTAT	Anti-sense	4
Rymo2F	8207-8226[c]	CTVGAGCAAGAACCATACAA	Sense	1
Rymo2R	8930-8949[c]	TYTGGIGAWGTICCATTWTC	Anti-sense	8
Tritimo1F	3578-3597[d]	GAGTTTCTTITWATIGGACA	Sense	4
Tritimo1R	4289-4311[d]	CCAAAITCIACIACAACGTCYGC	Anti-sense	2

[a] Positions of primers are given in reference to the genomic RNA 2 of *Soil-borne wheat mosaic virus* (NC_002041)

[b] Positions of primers are given in reference to the genomic beta RNA of *Barley-stripe mosaic virus* (X03854)

[c] Positions of primers are given in reference to the genome of *Ryegrass mosaic virus* (Y09854)

[d] Positions of primers are given in reference to the genome of *Wheat streak mosaic virus* (NC_001886)

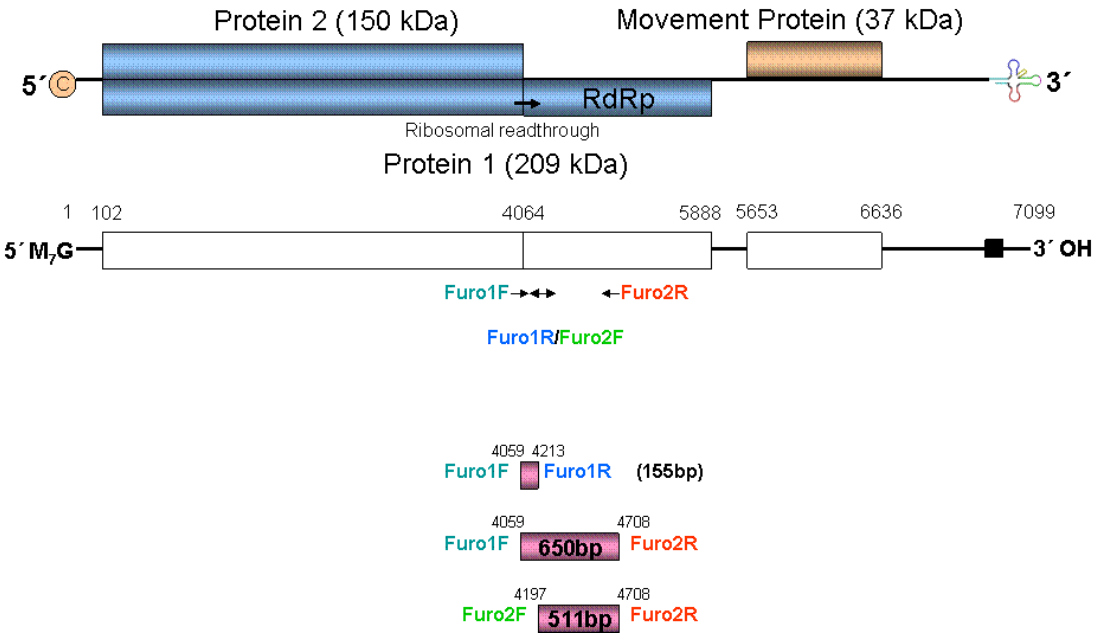

Figure 3.6 A schematic diagram of the RNA 2 from a furovirus genome (Soil-borne wheat mosaic virus; NC_002041) and the locations of four furovirus-specific universal primers

RT-PCR and cycling condtions

Three different primer combinations were used in the generic RT-PCR assays for furovirus detection (Table 3.15).

Table 3.15 Generic assays and their cycling conditions for furovirus detection

Primer combinations	Expected amplicon size (bp)	Annealing temperature (°C)	Extension time (seconds)
Furo1F-1R	155	45	20
Furo1F-2R	650	45	40
Furo2F-2R	511	45	40

Furovirus-specific universal primers evaluation using RT-PCR

Three assays using a combination of the different primers in reverse-transcription polymerase chain reactions (RT-PCR) were tested against seven known virus isolates from the *Furovirus* genus, representing 3 distinct furovirus species (Table 3.16). All three assays were able to detect 5/7 (71.43%) of the virus isolates tested and failed to detect samples 1 and 7, which were isolated from virus-infected plant sap stored on FTA cards.

Table 3.16 Name, origin and RT-PCR results of furovirus isolates tested using three generic assays specific for furovirus detection

Sample number	Virus isolate names	Geographical origin	Primer combinations		
			Furo1F-1R	Furo1F-2R	Furo2F-2R
1 [a]	Oat golden stripe mosaic virus (OGSV)	United Kingdom	-	-	-
2	Soil-borne cereal mosaic virus (SBCMV)	Germany	+	+	+
3	Soil-borne cereal mosaic virus (SBCMV)	New Zealand	+	+	+
4	Soil-borne cereal mosaic virus (SBCMV)	New Zealand	+	+	+
5	Soil-borne wheat mosaic virus (SBWMV)	Germany	+	+	+
6	Soil-borne wheat mosaic virus (SBWMV)	New Zealand	+	+	+
7 [a]	Soil-borne wheat mosaic virus (SBWMV)	United Kingdom	-	-	-

+ Positive detection; - Negative detection

[a] RNA isolated from FTA cards

Discussion

Although all three primer combinations amplified RNA from the same virus isolates, the amplicons produced by the primer pair 'Furo2F-2R' were brighter when viewed on ethidium-bromide stained agarose gel under UV light. There are also less non-specific binding of the primer pair 'Furo2F-2R' compared to the other two combinations, making the primer pair 'Furo2F-2R' the preferred combinations to be used in generic assays for furovirus detection. Optimisation of RT-PCR conditions is required for each primer pair, as well as cloning and sequencing of the amplicons produced to confirm the test result and virus isolate identity.

3.4.3.2 Hordeivirus

Positive virus controls

Three hordeivirus isolates were imported from Germany, NZ and UK (Table 3.20), which were all strains of BSMV. The hordeivirus isolates imported from Germany and New Zealand came in the form of extracted nucleic acids suspended in ethanol, whereas the virus isolate imported through UK was transported as plant sap spotted onto FTA cards (Whatman, UK).

Hordeivirus datasets

All nucleotide sequences of viral species in the genus *Hordeivirus* were retrieved from the public nucleotide sequence database Genbank. The sequences were collated and dated manually using their deposition dates in GenBank, but only full-length sequences were used for the purpose of universal primer design.

The 'hordeivirus' dataset contained three subsets, 'alpha', 'beta' and 'gamma', where each subset contained full-length sequences from the RNAα, RNAβ and RNAγ of the hordeivirus genomes respectively. The most comprehensive dataset containing either the most number of full-length sequences and/or representing the most number of distinct hordeivirus species is to be used for the purpose of universal primer design.

Identification of conserved sites within hordeivirus genomes

A total of eight full-length sequences from hordeivirus RNAβ were available in GenBank as of June 2009, representing four out of the five confirmed species from the genus *Hordeivirus* (Table 3.17). This dataset is more comprehensive than both the RNAα and RNAγ datasets, which consisted of seven and eight sequences of BSMV respectively, representing only one out of the four confirmed hordeivirus species. Full length sequence of *Anthoxanthum latent blanching virus* (ABLV) is not yet available.

Table 3.17 Names and acronym of confirmed species in the genus Hordeivirus**, full-length sequences and available positive virus controls**

Confirmed species	Acronym	Full-length sequences available in GenBank			Positive controls available
		RNAα	RNAβ	RNAγ	
Anthoxanthum latent blanching virus	ALBV	0	0	0	0
Barley stripe mosaic virus	BSMV	7	7	8	3
Lychnis ringspot virus	LRSV	0	1	0	0
Poa semilatent virus	PSLV	0	1	0	0
Total		**7**	**9**	**8**	

Hordeivirus-specific universal primer designs

The third, fourth and fifth ranked sites were chosen as target sites for the design of hordeivirus-specific universal primers (Figure 3.7; Table 3.18). The top two ranked sites were unsuitable for primer design due to the high probability of self-hybridisation.

Table 3.18 The rank, nucleotide positions, sequence contents, identification methods and N scores of conserved sites identified within the hordeivirus beta RNA full-length sequences

rank	nucleotide	nucleotide sequence of sites (5´ - 3´)	Identified	N

	positions[a]		by	scores[b]
1	2839-2858	AATAAGTATTGGCCWATWGT	NCSF[c]	0.10
2	2932-2951	GATAATATYCACAARTTYGC	NCSF	0.15
3	2836-2858[d]	CCNAATAAGTATTGGCCWATWGT	Eye	0.17
4	2941-2960[d]	CACAARTTYGCYAACGGWGG	Eye	0.20
5	2196-2215	GAYGARTAYACACTYGCYGA	NCSF	0.25
5	2271-2290	KTSGGKGAYGTMGCTCAAGG	NCSF	0.25
5	2202-2225[d]	TAYACACTYGCYGARAGYGCKGAA	Eye	0.25
6	391-410	CTWCCTGCTGMYDTWGACAG	NCSF	0.30
6	2559-2578	TGAWRYGCAAGGKWWAGAAT	NCSF	0.30
6	3645-3664	AAAMAAMAAAAARAAAARWR	NCSF	0.30
7	404-422	TWGACAGAMGNTTYGCHGG	Eye	0.42
8	376-395	GSGKTMRAKMRKGAYCTWCC	NCSF	0.50
9	172-191	YWWYHYRKYATGSCKAAYVT	NCSF	0.75
10	283-302	YHYKYAGAWRVYTGGVAHWM	NCSF	0.85
10	334-353	RRHGTYKYYWSMTCYMGDYS	NCSF	0.85

[a] Nucleotide positions given are in reference to an alignment of 8 hordeivirus β RNA full-length sequences

[b] Average nucleotide (N) scores of conserved sites within the hordeivirus β RNA

[c] Nucleotide conserved sequence finder (NCSF)

[d] Sites chosen for furovirus-specific universal primer design

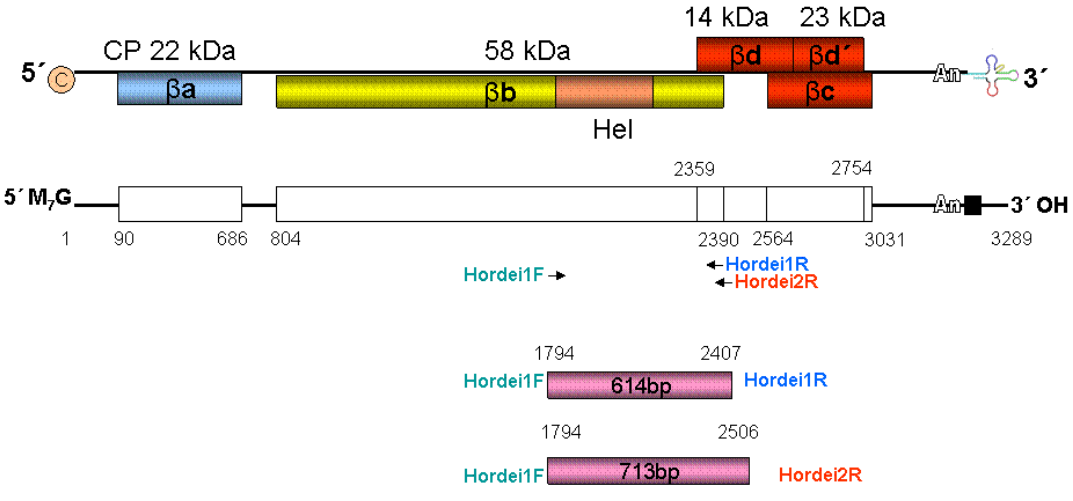

Figure 3.7 A schematic diagram of the RNAβ from a hordeivirus genome (Barley-stripe mosaic virus; X03854) and the locations of three hordeivirus-specific universal primers

RT-PCR and cycling conditions

Two generic RT-PCR assays were developed for the detection of hordeiviruses using a combination of three hordeivirus-specific universal primers (Table 3.14 and Table 3.19).

Table 3.19 Generic assays and their cycling conditions for furovirus detection

Primer combinations	Expected amplicon size (bp)	Annealing temperature (°C)	Extension time (seconds)
Hordei1F-1R	614	50	40
Hordei1F-2R	713	50	40

Hordeivirus-specific universal primers evaluation using RT-PCR

The two assays were tested against three virus isolates of *Barley stripe mosaic virus* (BSMV), one of the four confirmed species in the *Hordeivirus* genus (Table 3.3). Both assays were able to detect 2/3 (66.67%) of the virus isolates tested and failed to detect sample 3, which were isolated from virus-infected plant sap stored on FTA cards (Table 3.20).

Table 3.20 Virus isolate names, origin and RT-PCR results of two generic assays developed for hordeivirus detection

Sample number	Virus isolate names	Geographical origins	Primer combinations	
			Hordei1F-1R	Hordei1F-2R
1	*Barley stripe mosaic virus* (BSMV)	Germany	+	+
2	*Barley stripe mosaic virus* (BSMV)	New Zealand	+	+
3[a]	*Barley stripe mosaic virus* (BSMV)	United Kingdom	-	-

+ Positive detection; - Negative detection

[a] RNA isolated from FTA cards

Discussion

The primer combinations Hordei1F-1R and Hordei1F-2R both amplified RNA from two out of the three virus isolates tested. Hordei1F-1R is the recommended primer pair to use for

hordeivirus detection because this primer pair generated more PCR products than the primer pair Hordei1F-2R. The failed attempt at amplifying PCR products from RNA isolated from FTA cards is a recurring scenario that needs to be addressed. Spectrophotometer measurements of the RNA isolated from FTA cards indicate that the RNA is of sub-optimal quality (data not shown). The quality of the RNA isolated might also be affected by how the sample was prepared. Since the RNA was prepared overseas, the lysis buffer and method of the preparation might not have been ideal for storage on FTA cards. It is one of the aims of this project to quantify the effects of RNA storage on FTA cards. Upon completion of phase II of this project, we are hopeful that the results generated from these experiments will shed light on this problem.

To improve the primers, more positive virus controls are needed for testing. Due to the small size of the sequence database used in the design process, the primers designed at the present time might not match all possible variations that could exist in the hordeivirus genomes. It is envisaged that with the addition of more sequence data in the future, the primer sequence can be updated and become more reliable for hordeivirus detection.

3.4.3.3 Rymovirus

Rymovirus datasets

Nucleotide sequences from all members within the genus *Rymovirus* were retrieved from the public nucleotide sequence database Genbank. All sequences were dated manually using their deposition dates in GenBank before being separated into full-length and partial sequence datasets, as only full-length sequences were considered for the purpose of universal primer design.

Rymovirus-specific primer design

A total of four full-length sequences, representing all three confirmed species from the genus *Rymovirus* were available in GenBank as of June 2009. To date, there is no full-length sequence available for *Spartina mottle virus* (SpMV) in Genbank.

The RNA sequences were dated and sorted by their deposition date in GenBank before an alignment was created using the alignment program MUSCLE (Edgar, 2004). A total of 25 conserved sites identified using both the program NCSF and by eye were analysed using their rate of consensus decay. The sites were ranked based on their N scores and the top 10 ranked sites were accessed for their suitability as universal primers based on their sequence contents using OligoCalc (Kibbe, 2007). One of the top ranked, second ranked, fourth ranked and eigth ranked sites were chosen as targets for rymovirus detection (Figure 3.5; Table 3.22).

Table 3.21 The availability of full-length sequences available in GenBank for virus species in the genus Hordeivirus

Confirmed species	Acronym	Sequences available	Positive controls obtained
Agrypyron mosaic virus	AgMV	1	2
Hordeum mosaic virus	HoMV	1	0

Ryegrass mosaic virus	RGMV	2	2
Tentative species			
Spartina mottle virus	SpMV	0	0

Table 3.22 The rank, nucleotide positions, sequence contents, identification methods and N scores of conserved sites identified within the rymovirus genomes

Rank	Nucleotide positions[a]	Nucleotide sequence of sites (5´ - 3´)	Identified by	N scores[b]
1	8468-8487[c]	CTVGAGCAAGAACCATACAA	NCSF[d]	0.100
1	9489-9508	TTYGACTTCTAYGAAATAAC	NCSF	0.100
2	7611-7630[c]	AAGGTKGAAGCRAAYAAAAC	NCSF	0.150
2	8471-8490	GAGCAAGAACCATACAARWS	NCSF	0.150
3	2251-2269	AACMATGCATGTYGTKGAT	Eye	0.160
4	2249-2268	ARAACMATGCATGTYGTKGA	NCSF	0.200
4	3537-3556	AARTGCATYGCAACMWTTGT	NCSF	0.200
4	4434-4453	GTYGCRAGYTAYAATGAAGT	NCSF	0.200
4	4437-4456	GCRAGYTAYAATGAAGTKGA	NCSF	0.200
4	4570-4589	AAGCAYTTCATWGTYGCSAC	NCSF	0.200
4	7953-7952[c]	ATAGCAACWCCWGATGGDTC	NCSF	0.200
4	8309-8328	TACATACCWAWGYTSGAAAG	NCSF	0.200
4	9426-9445	AGRTAYATGCCWSGGTATGG	NCSF	0.200
4	9545-9564	GCAGATGAARGCYGCAGCHA	NCSF	0.200
5	4909-4928	GARCAAGCRMGARCYATGGC	NCSF	0.250
5	7284-7303	AARGAYVTCACWAAGTACGA	NCSF	0.250
5	3906-3925	GAATTYATAGTBCGWGGYGG	Eye	0.250
5	3912-3931	ATAGTBCGWGGYGGYGTTGG	Eye	0.250
5	4191-4210	GAYGARTGYCAYGTKATGGA	Eye	0.250
5	4591-4610	AACATYATYGARAAYGGYGT	Eye	0.250

Rank	Nucleotide positions[a]	Nucleotide sequence of sites (5´ - 3´)	Identified by	N scores[b]
6	8120-8142	CGRTAYTTTGCYAAYGGYGAYGA	Eye	0.261
7	7081-7102	AGTACACHTGGYTAACHAARTA	Eye	0.272
8	9252-9269[c]	GAWAATGGVACWTCMCCA	Eye	0.278
9	3927-3947	GTTGGVACHGGVAAATCAACA	Eye	0.286
10	8010-8033	CCRTCMACMGTSGTYGAYAAYACA	Eye	0.292

[a] Nucleotide positions given are in reference to an alignment of 4 rymovirus genomes

[b] Average nucleotide (N) scores of conserved sites within the rymovirus genomes

[c] Sites chosen for rymovirus-specific universal primer design

[d] Nucleotide conserved sequence finder (NCSF)

Positive virus controls

A total of four rymovirus isolates representing two distinct rymovirus species was imported from Germany, NZ and UK (Table 3.24). Rymoviru isolates imported from Germany and New Zealand came in the form of extracted nucleic acids suspended in ethanol, whereas virus isolates imported through UK were transported as plant sap spotted onto FTA cards.

Rymovirus-specific universal primers evaluation using RT-PCR

Two generic RT-PCR assays containing four universal primers were developed for rymovirus detection (Figure 3.5 and Table 3.14). The assays were tested against four virus isolates, representing two of the three confirmed species in the *Rymovirus* genus. Both assays were able to detect three out of the four virus isolates tested and failed to detect sample 3, which were isolated from virus-infected plant sap stored on FTA cards (Table 3.24).

Table 3.23 Generic assays and their cycling conditions for rymovirus detection

Primer combinations	Expected amplicon size (bp)	Annealing temperature (°C)	Extension time (seconds)
Rymo1F-1R	362	50	30
Rymo2F-2R	743	50	45

Table 3.24 Virus isolate names, origin and RT-PCR results of two generic assays developed for rymovirus detection

Sample number	Virus Names	Origin	Primer combinations	
			Rymo1F-1R	Rymo2F-2R
1	*Agropyron mosaic virus*	Germany	+	+

	(AgMV)			
2*	*Agropyron mosaic virus* (AgMV)	United Kingdom	-	-
3	*Ryegrass mosaic virus* (RGMV)	New Zealand	+	+
4*	*Ryegrass mosaic virus* (RGMV)	United Kingdom	+	+

+ Positive detection; - Negative detection

* RNA isolated from plant sap stored on FTA cards

Discussion

Although both primer pairs were able to produce amplicons of the right size from sample 4, amplicons produced by Rymo1F-1R were brighter when visualised on ethidium-bromide stained agarose gels under UV lights. Rymo1F-1R primer pair is therefore the preferred primers to be used for rymovirus detection. The rymovirus-specific primers are likely to only detect members of the *Rymovirus*, as they did not detect PVY, the closest relative to the rymoviruses.

Both primer pairs were able to detect AgMV imported in the form of purified RNA in ethanol (sample 1), but neither primer set were able to detect the UK isolate which was imported on FTA cards (sample 2). This supports previous results where generic assays designed for furoviruses and hordeiviruses have failed to amplify any RNA isolated from FTA cards. Interestingly, while both assays failed to detect sample 2, sample 4 was detected by both assays, albeit it too, was RNA isolated from FTA cards. This is the only positive virus control imported on FTA cards that tested positive using generic assays in this study.

3.4.3.4 Tritimovirus

Tritimovirus datasets

Nucleotide sequences from all members within the genus *Tritimovirus* were retrieved from the public nucleotide sequence database Genbank. All sequences were dated manually using their deposition dates in GenBank before being separated into full-length and partial sequence datasets, as only full-length sequences were considered for the purpose of universal primer design.

Tritimovirus-specific primer design

A total of three full-length sequences, representing all three confirmed species from the genus *Tritimovirus* were available in GenBank as of June 2009 (Table 3.25). The RNA sequences were dated and sorted by their deposition date in GenBank before an alignment was created using the alignment program MUSCLE (Edgar, 2004). A total of 35 conserved sites identified using both the program NCSF and by eye were analysed using their rate of consensus decay. The sites were ranked based on their N scores and two universal primers were designed to target the second and fourth ranked sites (Figure 3.8 and

Table 3.26). The sites were chosen based on their ranks, sequence contents and their close proximity to each other.

Table 3.25 The availability of full-length sequences available in GenBank for virus species in the genus Hordeivirus

Confirmed species	Acronym	Sequences available	Positive controls obtained
Brome streak mosaic virus	BstMV	1	0
Oat necrotic mottle virus	ONMV	1	0
Wheat streak mosaic virus	WSMV	1	3

Table 3.26 The rank, nucleotide positions, sequence contents, identification methods and N scores of conserved sites identified within the tritimovirus genomes

rank	nucleotide positions[a]	nucleotide sequence of sites (5´ - 3´)	Identified by	N scores[b]
1	3142-3161	GTACAACGARTTCWTCAACT	NCSF[c]	0.100
2	4644-4663	GACGTTGTSGTWGAYTTTGG	NCSF	0.150
2	4969-4988	TRTCGTAYATGGTKTGGATG	NCSF	0.150
3	4971-4995	TCGTAYATGGTKTGGATGRTKAACA	Eye	0.160
4	3923-3942	GAGTTTCTTRTWATDGGACA	NCSF	0.200
4	5961-5980	ATGCAATTTGGWMGRGAAGY	NCSF	0.200
4	7307-7326	TAYAAGGATGTRYTRAAGTA	NCSF	0.200
5	3154-3163	CWTCAACTCMWTTGGCTAYK	NCSF	0.250
5	3737-3756	TATGAYCATGADGCWACMCA	Eye	0.250
5	4004-4023	ATWTGYGAACCMACYCGMGT	Eye	0.250
5	4028-4051	GTMACMAAYTTGCADGATTCMATG	Eye	0.250
6	4653-4675	GTWGAYTTTGGBTWCAAGATYGT	Eye	0.261
6	7829-7851	GARTGGTTYATYGAYGCWGAYGG	Eye	0.261
6	8027-8049	AAYAAYAGTGGYCARCCRAGYAC	Eye	0.261
6	8420-8442	GTVGAAGCWTTYGGWTATGAYGA	Eye	0.261
7	7298-7316	GARGCDTAYTAYAAGGATG	Eye	0.263
8	2460-2481	GGATGGCVACMTWTTATGGDTG	Eye	0.273

rank	nucleotide positions[a]	nucleotide sequence of sites (5´ - 3´)	Identified by	N scores[b]
9	2471-2491	TWTTATGGDTGYTAYGAYGCA	Eye	0.286
10	3134-3153	SMKTGYGYGTACAACGARTT	NCSF	0.300
10	8045-8064	AGYACWGTTGTBGAYAAYAC	Eye	0.300
10	8345-8364	CGBATWGTRGCRATTCTKGA	Eye	0.300
11	2894-2913	YWSTTGRMAWCMARGMAGCG	NCSF	0.450
12	3095-3114	AARRAAWMWATRCTCGWMRR	NCSF	0.500
13	3206-3225	TATWCTGGYSYWGGMRSRMR	NCSF	0.550
14	3395-3414	ARYGYCWYRCARRTWWTRST	NCSF	0.600

[a] Nucleotide positions given are in reference to an alignment of 3 tritimovirus genomes

[b] Average nucleotide (N) scores of conserved sites within the tritimovirus genomes

[c] Sites chosen for tritimovirus-specific universal primer design

[d] Nucleotide conserved sequence finder (NCSF)

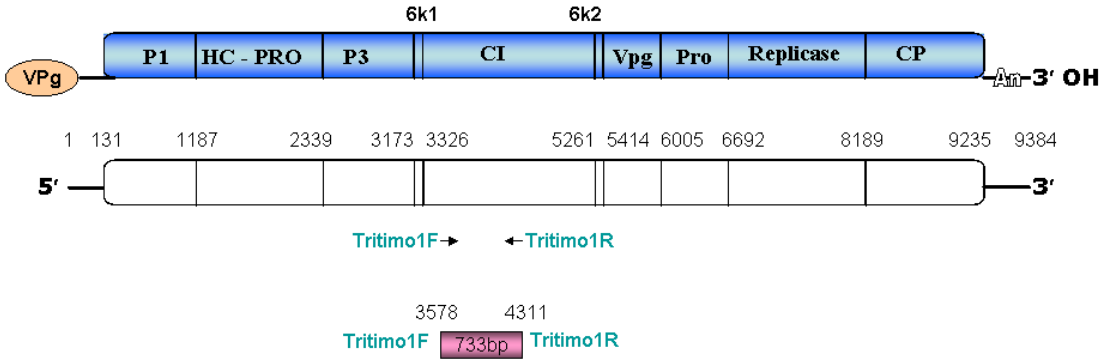

Figure 3.8 A schematic diagram of a tritimovirus genome (Wheat streak mosaic virus; NC_001886) and the locations of two tritimovirus-specific universal primers

Positive virus controls

A total of three tritimovirus isolates, all strains of *WSMV*, were tested. Two of which were imported from Germany and one is a local strain from Australia (Table 3.28). Tritimovirus isolates imported from Germany came in the form of extracted nucleic acids suspended in ethanol, whereas the virus isolate sourced from Australia was total RNA extract from a wheat sample infected with WSMV.

RT-PCR and cycling conditions

One generic assay was developed for the detection of tritimoviruses (Table 3.27). The assay was evaluated by applying it to three isolates of *Wheat streak mosaic virus*, the type species of the genus *Tritimovirus.*

Table 3.27 Cycling conditions for generic assay designed for tritimovirus detection

Primer combinations	Expected amplicon size (bp)	Annealing temperature (°C)	Extension time (seconds)
Tritimo1F-1R	733	48	45

Tritimovirus-specific universal primers evaluation using RT-PCR

The generic assay was able to detect all three isolates of WSMV (Table 3.28).

Table 3.28 Virus isolate names, origin and RT-PCR results of the generic assay developed for tritimovirus detection

Sample number	Virus isolate names	Geographical origins	Primer combinations
			Rymo1F-1R
1	*Wheat streak mosaic virus* (WSMV)	Germany	+
2	*Wheat streak mosaic virus* (WSMV)	Germany	+
3	*Wheat streak mosaic virus* (WSMV)	Australia	+

+ Positive detection; - Negative detection

3.5 Development of a quantitative molecular detection system to determine the rate of RNA degradation

Active monitoring of grain diseases in Australia plays an important role in post-entry quarantine, and large scale surveys are often conducted to support Australia's disease-free status. Sample preparation is often the bottleneck in these surveys, as they can be time-consuming and laborious, limiting the number of samples that can be collected and processed. In times of an incursion, when it is possible that virus-infected plant materials cannot be moved off site and/or on-site high volume processing is required, sample preparation becomes an important issue for quarantine officers. There is a need for faster collection and processing of infected plant materials, especially in times of crisis, to enhance Australia's capability for plant disease management and incursion response. FTA card technology also has the potential to provide a safe and efficient method of transporting quarantine material between states and countries.

Reports of using FTA (Flinders Technology Associates) cards for the transport and storage of plant virus RNA extracts exist in the literature (Natarajan *et al.*, 2000; Rogers *et al.*, 2000; Ndunguru *et al.*, 2005; Perozo *et al.*, 2006; Purvis *et al.*, 2006; Picard-Meyer *et al.*, 2007). FTA cards were initially developed to allow rapid extraction, transport and storage of DNA from an array of organisms (Hopkins, 1977; Devost *et al.*, 2000; Dobbs *et al.*, 2002; Moscoso *et al.*, 2004; Moscoso *et al.*, 2005; Borman *et al.*, 2006; Fujita *et al.*, 2006; Jaravata *et al.*, 2006; Fontaine *et al.*, 2007; Moro *et al.*, 2007; Moscoso *et al.*, 2007). The use of FTA cards could potentially reduce the complexity and time required for sample collection and processing, enabling large amount of plant materials to be sampled in field surveys and in incursion responses. However, there is little information on the quality of the RNA extracts and the duration in which the RNA can be stored on the FTA cards. Given the potential benefits FTA cards have over traditional sample collection and processing, the feasibility of using them in plant virus RNA transportation and storage needs to be investigated.

Conventionally, purified viral RNA extracts are stored at -20°C until use and are usually subjected to frequent freeze-and-thaw cycles. The freeze-and-thaw cycles can potentially shear the RNA, resulting in poor integrity of the RNA extracts. One way to circumvent this problem is to store the total RNA extracts as aliquots to minimise the number of freeze-and-thaw cycles they are subjected to. It would be interesting, however, to determine the impact on total RNA integrity due to the freeze-and-thaw cycles. To do so, a quantitative molecular system capable of determining the rate of degradation for RNA is required.

In this project, a molecular based quantification system capable of quantifying the amount of RNA will be developed to determine the length of which purified total RNA extracts and plant sap can be stored on the FTA cards.

3.5.1 Experimental Design

Both purified RNA extracts and homogenized plant sap will be spotted onto the FTA cards and stored under different conditions (Figure 3.9). RNA will be isolated from the FTA cards at regular time intervals and the amount of RNA will be measured using a RT-qPCR system. The amount of amplifiable RNA will be measured over time and used as an indicator of the integrity of the RNA stored on the FTA cards.

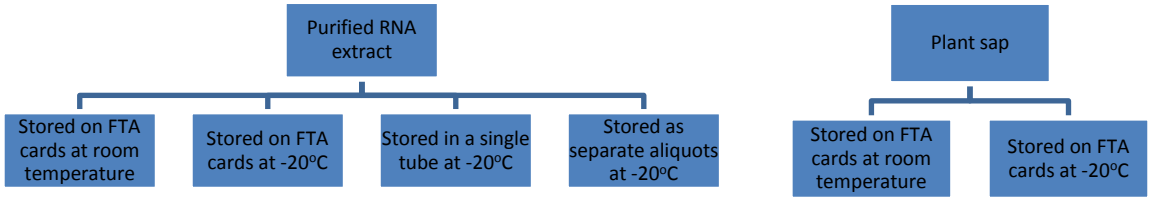

Figure 3.9 Storage conditions of viral RNA for FTA experiment

3.5.1.1 Variables and parameters of the detection system

To develop a detection system that can accurately quantify the amount of usable (amplifiable) RNA stored on FTA cards, the following parameters must be considered and addressed.

- **Standards :** A standard curve is required using transcribed ssRNA of the target sequence. The transcribed ssRNA needs to be DNA-free and have a concentration range that covers that of the isolated RNA samples. The purpose of the standard curve is to provide a baseline from which all measurements of the target RNA is compared to. It is absolutely essential that the standards are of high quality and stored appropriately to avoid degradation.

- **Amount of template in each reaction:** There are three important check points in ensuring that the amount of amplifiable RNA stored on the FTA cards after a timed period is accurately quantified.

  Checkpoint 1: each aliquot spotted onto the FTA cards must be identical, which can be difficult due to human errors associated with pipetting.

  Checkpoint 2: when isolating RNA from the FTA cards, each spot must be processed in its whole entity to make sure the same amount of RNA is being isolated.

  Checkpoint 3: each RNA processing procedure must be identical to ensure the similar amount of RNA is being isolated from the FTA cards.

- **Reverse transcriptase (RT) enzyme efficiency:** RT enzyme is known for its variable efficiency in RT-PCR and accounts for most variance observed between the replicates of a RT-PCR. This variation must be taken into account when attempting RNA quantification using reverse-transcription quantitative polymerase chain reaction (RT-qPCR).

3.5.2 Quantitative RT-PCR detection system development

3.5.2.1 Construction of RNA standard curves

Potato plants infected with the NTN strain of PVY were grown and maintained in the glasshouse. A set of primers PVYoutF, 5'- TTCACTGATGCAGATAAGGAAGAAAT -3' and PVYoutR, 5'- GTTGTGACATTCTATCGAGAATGCTC -3' was used to amplify a ~680 bp fragment of the NIb region of the PVY genome. This fragment was then cloned and sequenced and the verified clone used as a template for downstream transcription of PVY RNA. The transcribed RNA is used to construct the RNA standard curves. The quality and quantity of the transcribed RNA will be determined using the spectrophotometer Nanadrop (Thermo Fisher Scientific Inc, USA) prior to use. RNA transcripts will be treated with DNases to ensure they are free of DNA. To minimise the risk of degradation, the transcribed RNA will be stored as 20µl aliquots at -70^{o}C to avoid frequent freeze and thaw.

3.5.2.2 The RT-qPCR primers and probe

A set of internal primers, PVY-F and PVY-R, along with an internal hybridisation probe specific to this strain of the PVY were designed from within the region amplified by the PVYoutF and PVYoutR primers (Figure 3.10; Table 3.29). The PVY specific probe (PVY-probe) is dual-labelled with FAM at one end and BHQ-quencher at the other. An RT-qPCR assay utilising the primers and probe will be developed to detect the quantity of viral RNA isolated from the FTA cards.

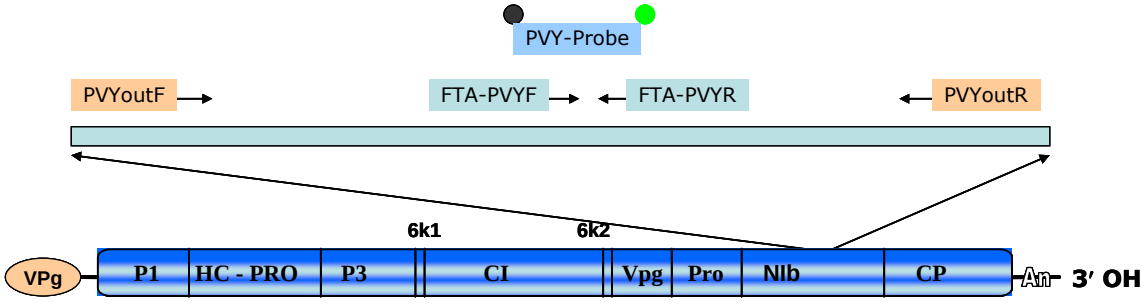

Figure 3.10 A schematic representation of the PVY genome and locations of the primers and probes designed for the RT-qPCR assay for PVY RNA detection

Table 3.29 Primer sequences, annealing temperatures and the expected amplicon sizes of the RT-qPCR assays

RT-qPCR assays	Primers	Primer Sequences 5'-3'	Tm (°C)	Expected Amplicon Size (bp)
FTA PVY	PVY-F	GCTGGACTGTTGGAATGACTAAG	53	120
	PVY-R	ATGGGGTGAGTGAACTATCGAA		
	PVY-probe	ACCTGAAAATTGGGTGTACTGCGATG		
pTRI-Xef	Xef-F	AGGATCTGGCAAAGTCACAAA	53	239
	Xef-R	CTAATAATCTAAAACTGCCACAAA		
	Xef-probe	CTCAGAAAGCAGCGAAAACCAAATG		

3.5.3 Discussion

The RT-qPCR detection system, once developed, will provide a powerful tool to answer some very important questions raised in phase I of this project in regards to the use of FTA cards for viral RNA transport and storage. By measuring the amount of amplifiable RNA stored on FTA cards, its rate of degradation can be determined.

There are several important considerations that need to be addressed in the development of this system; each of which will take a significant amount of time to resolve. One of the most prominent technical issues with the development of this detection system is the generation of reliable standards in the form of DNA-free RNA transcripts. The DNA is difficult to remove and successive DNase treatment of the RNA transcripts resulted in RNA of poor quality and low concentrations. Reproducibility of the experiment is another issue where pipetting errors can lead to inaccurate quantification of amplifiable RNA. An automated system of pipetting should be used, either through the use of an electronic pipette or a stepper pipette. The development of a multiplex assay will also take a significant amount of time due to optimisation. Phase II of the project will see the completion of an optimised multiplex Rt-qPCR assay for the quantification of PVY RNA stored on FTA cards.

3.6 Documented clear directives for PHASE II (2009-2011)

To deliver on this activity a three year research proposal was produced and submitted to the CRCNPB for funding. This project proposal was approved for 12 months funding with a strong recommendation to seek funding from GRDC for a further two years.

A summary of the project proposal is presented below:

Project title: Improved Post Entry Quarantine Diagnostics

Project Aim

Develop a new diagnostics platform for post entry plant quarantine to support the detection of emergency plant pests in the Australian grains and nursery industries.

Background:

- International germplasm movement is increasing and current estimates are that annually 23 tons of wheat germplasm is shipped globally.

- The Australian Quarantine and Inspection Service (AQIS) facilitates the importation of over 500 horticultural cultivars, 2,000 seed lines and 500 high risk and 70,000 medium risk ornamental plant lines/cultivars each year.

- Plant material imported for propagation is visually inspected and appropriately fumigated to eliminate insect pests and fungal pathogens. The remaining quarantinable pests and diseases are the obligate pathogens such as the viruses and some bacteria.

- Recent research (CRCNPB 40050) has designed a diagnostic test that can reliably detect a group of viruses (the potyvirus genus).

- Additional tests have been designed for four virus genera that are important for the grains industry; furoviruses, hordeiviruses, tritimoviruses and rymoviruses.

- At the completion of Phase II of this project we will be able to detect one third of all known plant viruses which includes 14 of the 22 viruses listed under the *Emergency Plant Pest Response Deed* (EPPRD).

This project aligns with the CRCNPB strategic plan in Impact Management (Develop tools to underpin optimal response strategies, area freedom protocols and pre-emptive crop management strategies) and Diagnostics (Develop new, robust, cost-effective diagnostic tools for accurate identification of emergency plant pests).

This project aligns with GRDC strategic plan to 'Practice strategies relating to biosecurity and practical integrated pest management,' and the GRDC strategy to support CRCNPB projects in the diagnostics, preparedness and prevention programs.

Project Objectives:

- Design, develop and validate diagnostic protocols for groups of viruses that include EPPs of importance for the Australian grains and nursery industries.
- Document a strategy for group-specific primer design
- Identify a process for ratification of group-specific tests via SPHDS
- Train PEQ pathologists and relevant plant diagnosticians in Australia and New Zealand in the use of the diagnostic protocols for the detection of virus genera.
- Conduct a QUADS workshop on post entry quarantine diagnostics and procedures.

Project Outcomes

Industry

- The outputs of this project will increase the capacity of Australia to detect EPPs that threaten our grains and horticultural industries. The recent incursion of WSMV cost the grains industry millions of dollars and the ongoing management of this disease is considerable in central and southern New South Wales (NSW).

- A recent meeting between representatives from the plant importing industries and AQIS (Post Entry Plant Industry Consultative Committee meeting in Sydney, October 2008) identified reducing time in PEQ as a key priority.

- Many of the existing PEQ disease screening protocols are based on visual assessments and/or biological indicator systems which although reliable are often quite time consuming.

Research:

This project will transform PEPQ disease diagnostics through the development of:

- Advanced design strategies for group-specific primers.
- Validated diagnostic protocols for the detection of 15 plant virus genera.
- Improved methodologies to screen field collected samples for viruses
- A capacity to detect both known and unknown viruses.
- Refereed publications
- Plant Health Committee endorsed national diagnostic protocols

3.7 Presentation and Publications

Findings from this project have been communicated to other colleagues and peers in the forms of peer-reviewed journal article and conference presentations. A list of publications follows:

Journal article

L. Zheng, B. C. Rodoni, M. J. Gibbs and A. J. Gibbs (2009). A novel pair of universal primers for the detection of potyviruses. Plant Pathology Published Online: 8 Dec 2009 Early view online Doi: 10.1111/j.1365-3059.2009.02201.x.

Conference presentations

L. Zheng, B. C. Rodoni (2010) – Diagnostic tools to support quarantine pathology laboratories. Global Biosecurity 2010 (Brisbane, Australia) – Oral Presentation

L. Zheng, A. Freeman, G. Clover, J. Thomas, K. Davis, M. Whattam and B. Rodoni (2009) – Grains Post Entry Quarantine: A review. Science Exchange 2009 (Sunshine Coast, Australia) – Oral Presentation

L. Zheng, M. Gibbs and B. Rodoni (2009) – Assessing the apparent stability of conserved sites in virus genomes over time. 7th Asia Pacific Bioinformatics Conference (Beijing, China) – Poster Presentation

L. Zheng, M. Gibbs and B. Rodoni (2008) – A pair of degenerate primers for potyvirus detection: from design to application. 8th Australasian plant virology workshop (Rotorua, New Zealand) – Oral Presentation

Reports

B. Rodoni, L. Zheng (2009) - A review of the legal importation of grains material into Australia and New Zealand: Identification of critical control points to better manage biosecurity risks. Report submitted to the CRC for National Plant Biosecurity

4 Implications for stakeholders

Failure in Post Entry Quarantine systems has significant consequences for plant industries and this was clearly demonstrated with the incursion of *Wheat streak mosaic virus* (WSMV) in 2003. This incursion has been estimated to have cost the Australian grains industry $10 million and WSMV damage has been reported to be as high as 80% in some crops in central and Southern NSW. Significantly, the economic effects of the presence of WSMV in Australia are felt on an annual basis via reduction in crop yields and the costs of implementing a disease management strategy. *Soil-borne wheat mosaic virus* (SbWMV) has recently been detected in New Zealand and therefore is a high risk for Australia. SbWMV is a furovirus that can cause up to 70% yield loss in susceptible cultivars and is extremely difficult to control once the virus and its fungal vector is established in the soil. It is critical that PEQ keeps this pathogen and associated exotic viruses out of Australia.

The review of the importation processes of winter cereals into Australia and New Zealand conducted as a part of this project identified a need to review the current 'open quarantine' policy, and develop diagnostic tools to support PEQ pathologists. PEQ stations currently rely on a visual inspection of cereal germplasm to identify symptoms of viral diseases. Visual inspections can be subjective and distinguishing between viruses that cause similar symptoms is difficult. As the first defence point against EPP incursions, it is critical that the diagnostic protocols developed in Phase I (potyvirus protocol) and the fourteen additional plant virus genus protocols being developed in Phase II are applied on germplasm material that enters Australia through PEQ stations.

The outputs of Phase I and Phase II of the CRCNPB/Vic DPI/GRDC project will benefit PEQ stations in Australia, New Zealand and possibly other members of the QUADS alliance. A key activity of Phase II is to engage quarantine pathologists from QUADS country participants. It is hoped that the protocols developed in Phase I and II of this project will be adopted in diagnostic labs in the region. Additionally, plant pathologists based at the International Centre of Agricultural Research in Dryland Areas (ICARDA) and the International Maize and Wheat Improvement Centre (CIMMYT) will also be informed of the developed protocols. It is estimated that more than 20 tonnes of germplasm is moved between institutes per annum at a global level and the bulk of this material originates from CIMMYT and ICARDA.

There is a requirement for agricultural industries in Australia to work with AQIS and ensure that pest lists are kept up-to-date as this ensures that imported germplasm will be screened for new and emerging diseases at the point of entry. It is imperative that the protocols developed in this project are properly validated in Phase II of the project and that the protocols are maintained and updated on an on-going basis. This will ensure that world's best practice procedures are used to screen germplasm entering Australia and result in improved biosecurity for our agricultural industries.

Critical to the success of developing, validating and maintaining diagnostic protocols for the detection of EPPs is access to safe positive controls. Strict restrictions are currently enforced by Australian quarantine to limit the importation of positive controls as virus infected plant tissue is deemed to great a risk to Australian agriculture. To facilitate the easy and safe importation of positive controls for use in PCR we have initiated an evaluation of the use of using sap extracts stored on FTA cards. Not only does this technology provide the potential to denature plant viruses in infected sap, it is also extremely easy to prepare the samples at the point of origin. The evaluation of the FTA cards will continue into Phase II of this project.

5 Recommendations

The following recommendations are based on the findings from this 18 month project Considerable progress has been made in understanding the limitations of the current importation procedures, particularly for winter cereals, into Australia, and in developing diagnostic tests that can detect both known and unknown viruses. To maximise the benefits of this research to industry we recommend the following:

1. The CRCNPB work with the grains industry and AQIS to review the current open quarantine policy.

2. The grains industry work with AQIS to ensure that the AQIS ICON database is regularly updated (at least once a year).

3. The group specific primers for the detection of potyviruses be used on a routine basis in Australian plant diagnostic laboratories for the detection of potyviruses.

4. The CRCNPB support the funding of Phase II of this project to ensure the validation of the group specific primers for the furoviruses, hordeoviruses, rymoviruses and tritimoviruses and the development of protocols to detect a further 10 genera of plant viruses.

5. Expand the outputs of this project to target diagnostic support in PEQ for the nursery industry and to target the development of diagnostic protocols for higher organisms (e.g. bacteria).

6. Continue the validation of diagnostic protocols and develop a collection of safe positive control extracts to support the diagnostic tests. It is recommended that a system be developed that actively updates and modifies group-specific primers on an on-going basis.

7. The CRCNPB foster partnerships with AQIS to train and up-skill plant pathologists in the developed tests.

8. The CRCNPB develop partnerships with both New Zealand MAF and other QUAD partners to improve the diagnostic tools available for quarantine pathologists at a regional level.

6 Abbreviations/glossary

ABBREVIATION	FULL TITLE
AQIS	Australian Quarantine and Inspection Service
cDNA	Complementary deoxyribonucleic acid
CRCNPB	Cooperative Research Centre for National Plant Biosecurity
CP	Coat protein
dI	Deoxyinosine
DPI	Department of Primary Industries
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPP	Emergency plant pest
FTA	Flinders technology associates
ICON	AQIS's import conditions database
ISEM	Immunosorbent electron microscopy
MAF	Ministry of Agriculture and Forestry
MUSCLE	Multiple sequence comparison by log-expectation
NCSF	Nucleotide conserved site finder
NZ	New Zealand
ORF	Open reading frame
QUADS	The Quads group of countries comprise Australia, Canada, the US, and NZ that meets annually on a formal basis to discuss common approaches to Animal Health issues of international significance
PEQ	Post entry quarantine
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
WSMV	Wheat streak mosaic virus

7 Plain English website summary

Please complete table using plain English. This information will be published on CRCNPB's website for a public audience.

CRC project no:	CRC0050
Project title:	Enhanced diagnostic platforms for Post Entry Quarantine (PEQ) and Market Access
Project leader:	Brendan Rodoni
Project team:	Linda Zheng, Gerard Clover
Research outcomes:	In phase I of this project the project team i) conducted a review of the importation processes of cereals into Australia and New Zealand ii) designed and validated molecular tests that target groups of plant viruses that pose a threat to the grain's industry and iii) investigated the application of the Flinders Technology Associates (FTA) card technologies for rapid and safe collection of plant virus samples, as well as storage of viral ribonucleic acid (RNA).
Research implications:	This project has identified some areas for improvement for the importation of winter cereals into Australia. A superior diagnostic tool for the detection of potyviruses, the largest plant virus genus, has been developed and validated. A further four plant virus genus-specific tests have been designed and are currently being validated. Importantly this project has generated a foundation base for the design, development and validation of quality ready-to-go diagnostic tools to be used by quarantine pathologists. Strategies for the safe transportation of positive controls have also been initiated as a result of this project.
Research publications:	**Journal article** L. Zheng, B. C. Rodoni, M. J. Gibbs and A. J. Gibbs (2009). A novel pair of universal primers for the detection of potyviruses. Plant Pathology Published Online: 8 Dec 2009 Early view online Doi: 10.1111/j.1365-3059.2009.02201.x. **Conference presentations** L. Zheng, B. C. Rodoni (2010) – Diagnostic tools to support quarantine pathology laboratories. Global Biosecurity 2010 (Brisbane, Australia) – Oral Presentation L. Zheng, A. Freeman, G. Clover, J. Thomas, K. Davis, M. Whattam and B. Rodoni (2009) – Grains Post Entry Quarantine: A review. Science Exchange 2009 (Sunshine Coast, Australia) – Oral Presentation L. Zheng, M. Gibbs and B. Rodoni (2009) – Assessing the apparent stability of conserved sites in virus genomes over time. 7th Asia Pacific Bioinformatics Conference (Beijing, China) – Poster Presentation L. Zheng, M. Gibbs and B. Rodoni (2008) – A pair of degenerate primers for potyvirus detection: from design to application. 8th Australasian plant virology workshop (Rotorua, New Zealand) – Oral Presentation **Reports** B. Rodoni, L. Zheng (2009) - A review of the legal importation of grains material into Australia and New Zealand: Identification of critical control points to better manage biosecurity risks. Report submitted to the CRC for National Plant Biosecurity

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Appenidx A: A review of the legal importation of grains material into Australia and New Zealand: Identification of critical control points to better manage biosecurity risks

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A review of the legal importation of grains material into Australia and New Zealand: Identification of critical control points to better manage biosecurity risks.

By

Brendan Rodoni and Linda Zheng

(June 2009)

Appenidx B: A novel pair of universal primers for the detection of potyviruses

(Double click to access document below)

Plant Pathology (2009) Doi: 10.1111/j.1365-3059.2009.02201.x

A novel pair of universal primers for the detection of potyviruses

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A novel pair of universal primers was developed to detect potyvirus species after conserved sites were identified using all full-length potyvirus sequences available by 2005. The breadth of specificity of the new primers, NIb2F and NIb3R, was investigated and compared with the specificity of two routinely used primer pairs in plant virus diagnostic laboratories. RNA from 40 potyvirus isolates representing 23 recognized and three possible new species was tested. Reactions with NIb2F and NIb3R produced amplicons of 350 bp from all 40 virus isolates tested. Reactions with the previously published WCIEN and Potyviral primers amplified cDNA from 32 and 21 isolates, representing possibly 21 and 15 species, respectively. The identity of 12 unknown potyvirus isolates was confirmed by sequencing and three were found to be potentially distinct potyvirus species. Gel banding patterns from reactions with NIb2F and NIb3R were simpler to interpret than those from reactions with the other two primer sets; fewer products were visible and the cDNA fragments were less variable in size. RT-PCR with the novel primers is predicted to be able to detect virus isolates from all major groups within the genus *Potyvirus* and its reliability makes it well suited for use as a routine diagnostic assay.

Keywords: biosecurity, degenerate primers, detection, diagnostics, plant virus, potyvirus

Introduction

The genus *Potyvirus* is one of the largest virus genera and currently includes 111 confirmed species; a further 86 tentative species have been noted by the International Committee on Taxonomy of Viruses (ICTV; Fauquet *et al.*, 2005) and several new potyvirus species are discovered annually (Zheng *et al.*, 2008b). Potyviruses are one of the most economically important groups of plant viruses and pose a threat to crops around the world (Dujovny *et al.*, 2000; Moriones & Luis-Arteaga, 2000; Inoue-Nagata *et al.*, 2002; Larsen *et al.*, 2003).

Since the early 1990s, at least seven attempts have been made to design universal primers to detect all potyviruses (Langeveld *et al.*, 1991; Nicolas & Laliberte, 1991; Colinet & Kummert, 1993; Pappu *et al.*, 1993; Rossolini *et al.*, 1994; Gibbs & Mackenzie, 1997; Chen & Adams, 2001; Ha *et al.*, 2008). Universal primers for virus genera are often highly degenerate and designed by finding conserved sequences in an alignment by eye. They permit the detection of a range of species with different sequences as well as previously unknown viruses. Universal primer design depends on the sequence data of the viruses available at the time, so primers developed using a small sample of sequences may not cover variations of unknown species in the virus group. An analysis of 17 of the most conserved sites within potyvirus genomes revealed the decay in their consensus as new sequences became available, resulting in an apparent loss of stability (Zheng *et al.*, 2008b). The most conserved site identified in that study was in the region encoding the nuclear inclusion protein b (NIb) and had an average nucleotide variant score (N score) of 0·55. The site encoded the CVDDFN motif and was never targeted previously by primers designed for potyvirus detection. The GNNSGQ motif located at the centre of the NIb gene, which was targeted by universal primer Potyviral 2 (Gibbs & Mackenzie, 1997) was ranked the seventh most conserved site (N score of 0·85), whilst the WCIEN motif, located in the coat protein (CP) region of the potyvirus genome, which was targeted by the CN48 primer (Pappu *et al.*, 1993) was ranked the 12th most conserved site (N score of 1·10) amongst the 17 sites (Zheng *et al.*, 2008b) (Fig. 1).

In this study, a pair of universal primers, NIb2F and NIb3R, was developed based on two conserved sites in the NIb region of potyvirus genomes that were chosen using the Nucleotide Conserved Site Finder (NCSF) program and the N score measures. The NIb2F and NIb3R primer sites were ranked first and ninth, respectively (Zheng *et al.*, 2008b). The breadth of specificity of this

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